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The relative sensitivity of algae to inhibitors from plant litter

A thesis submitted for the degree of Doctor of Philosophy

by

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April 1999

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Abstract

Decomposing barley straw (*Hordeum vulgare*) and oak leaves (*Quercus robur*) have previously been shown to inhibit the growth of a limited number of algae and cyanobacteria. Bioassays were conducted on a range of algae and cyanobacteria to evaluate their relative sensitivities to litter-derived inhibitor(s). A range of sensitivities were found, including some species that were stimulated by litter-derived inhibitor(s). Susceptibility to decomposing plant litter did not appear to be related to general taxonomic or structural features since susceptibility differed widely, even amongst members of the same genus. A microcystin-producing strain of *Microcystis aeruginosa* was very susceptible to decomposing barley straw.

No specific effect on cell structure or morphology could be attributed solely to the litter-derived inhibitor(s). Evidence suggested that cell division, rather than cell expansion, was slowed or inhibited. Bioassays using *Euglena gracilis* showed the inhibitory compounds were not derived from the phototransformation of litter decomposition products and were not acting primarily by inhibiting photosynthesis.

Barley straw inhibited the growth of filamentous algae in a drainage channel and, subsequently, the channel was recolonized by macrophytes. A one-year treatment with barley straw inhibited algal growth, but this reduction in growth was not maintained when the straw was removed. After three-years treatment with barley straw, macrophytes retained dominance after the removal of the straw. No inhibition of algal growth was observed in a different straw-treated drainage channel. The implications for water management are discussed.

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Preface

Throughout this thesis I have used the word algae to include both the eukaryotic organisms and the prokaryotic cyanobacteria, unless otherwise stated in the text. Although phylogentetically an unnatural grouping, historically the two types have been grouped together and the term 'algae' is often still used in general texts.

The classification of the diverse group comprising the 'algae' is presently in a state of flux and presents a problem for taxonomic nomenclature. More recently it has been popular to classify the eukaryotic algae as belonging to the kingdom Protoctista (Margulis & Schwartz, 1998). However, as Hoek *et al.* (1995) point out, this kingdom and the classification system it is based upon is not phylogenetically coherent, consequently it is as artificial as more traditional classifications where algae are grouped with the 'plants'. Although some eukaryotic 'algae' would undoubtedly be better placed in the kingdom Protoctista, for reasons of consistency the classification of Hoek *et al.* (1995) is used throughout the entire thesis.

The current work also only focuses on temperate freshwater-bodies and no attempt has been made to take examples from tropical, arctic or boreal regions.

CHAPTER 1

Introduction

1.1 Eutrophication and its associated problems

Eutrophication is the increase of organic matter production in waters in relation to increasing nutrient supplies. Eutrophication can occur naturally in a waterbody through enrichment and siltation caused by natural weathering processes, when it is termed natural eutrophication (Reynolds, 1984a; Boney, 1989); or it can be caused by disturbance of the natural ecosystem via human influences, when it is termed cultural eutrophication (Reynolds, 1984a). Humans have introduced large amounts of nutrients into waterbodies through sewage discharges and farmland fertilizer run-off, thus increasing the resources available for algal growth and leading to increases in algal biomass. In temperate freshwaters, phosphorus is normally the limiting nutrient for the growth of most algae (Reynolds 1984a; Harris, 1986) since the amount of biologically available phosphorus is small in relation to the quantity required for algal growth (Canter-Lund & Lund, 1995). Nitrogen can also be limiting where high amounts of phosphorus are present and it is an increase in these two nutrients which is usually the main cause of eutrophication and the associated algal growth (Reynolds, 1984a, 1984b).

There are many problems associated with increased growths of algae. At high nutrient concentrations, only very competitive macrophytes such as *Ceratophyllum demersum* L. and *Potamogeton pectinatus* L. may be able to survive but, eventually, even these may be suppressed as algae become dominant (Moss *et al.*, 1996a) (see chapter 5). The resultant loss of macrophytes can have implications for the fauna of a water body since macrophytes normally provide food, habitat and refuge for small invertebrates and some species of fish. Dense growths of filamentous algae such as *Hydrodictyon reticulatum* (L.) Lagerheim and *Cladophora glomerata* (L.) Kütz also look unsightly and cause amenity problems, becoming tangled around boat propellers and fishing lines. For

example, *Hydrodictyon reticulatum* is considered a serious economic nuisance of recreational waters in New Zealand (Hawes & Smith, 1993). When the filaments die there are more problems as the rotting process can cause deoxygenation of the water, which may lead to fish kills and the release of unpleasant odours. Excessive growths of algae can interfere with filters at water treatment works through the build up of slime films and may cause undesirable tastes and odours in potable water (Palmer, 1980). Nuisance growths of cyanobacteria are particularly undesirable since certain species or strains can produce toxins; the production of such toxins can lead to reservoirs being closed to water extraction and the closure of amenity waters. Cyanobacterial blooms can cause illness and death in farm animals (Mez *et al.*, 1997), and skin irritations, allergic responses and even death in humans: in Brazil in 1996 deaths amongst haemodialysis patients were attributed to the presence of cyanobacterial toxins in the water used for dialysis (Dunn, 1996). Overall, the human implications and ecological effects of cultural eutrophication and the concomitant increase in algal biomass are perceived as undesirable. The presence of algal blooms, especially of toxic cyanobacteria, is well reported in the media, heightening public awareness to the problem and increasing the pressure on environmental agencies and water companies to do something about them.

1.2 What makes a particular species of algae bloom?

There is no definition as to how large algal biomass must be before it is considered to be a bloom, although Horner *et al.* (1983) suggested that periphytic algae becomes a nuisance when it covers greater than 20% of a substratum. It could be argued that the term 'bloom' could include the annual increase in biomass of algae which is achieved during the growth season, even in nutrient-poor waterbodies. For the purposes of this study a bloom of algae will be the point at which the amount of biomass attained in some way becomes a nuisance to mankind. This may not necessarily mean that all so-called nuisance blooms are caused by humans and can include those which occur via natural eutrophication.

The seasonal cycle of algal biomass in temperate waters is determined primarily by changes in light and temperature (Reynolds, 1984a; Harris, 1986; Round, 1984). Accompanying the seasonal effects are other environmental processes which control algal growth and species succession, such as nutrient availability and interactions with other aquatic organisms.

1.2.1 Gain and loss processes

The algal biomass that can be attained is a balance between the gain (growth) and loss processes which occur in the environment (Reynolds, 1984a; Harris, 1986). A gain process is the net growth and increase in biomass of algae, which is controlled largely by light, temperature and the availability of nutrients. Reynolds (1984a) defined a loss process as any process which actively removes biomass from the part of a water body under consideration and therefore depletes the potential stock of growing organisms. It is the loss processes which appear to be the driving variables controlling species succession. Loss processes can be biotic (e.g. grazing and parasitism) or abiotic, with algal biomass being lost through sedimentation and hydraulic washout amongst other things.

Much work has been carried out to try and understand the effects that gain and loss processes have upon algae, especially phytoplankton. The theory of 'r' and 'K' selection developed by MacArthur and Wilson (1967) has been applied to phytoplankton to try and explain the relative advantages/disadvantages that different species have under defined conditions (Sommer, 1981; Kilham & Kilham, 1980). 'r' selection favours short-lived, fast-growing species which invest in relatively greater reproductive effort. In 'K' selection proportionately more resources are diverted to non-reproductive activities, favouring organisms resistant to loss processes and efficient in the utilization of resources in short supply. Reynolds (1983) went on to add a third category, 'w', to explain those species tolerant of disturbance. This concept has been applied by Reynolds (1988a) to approximate to the C, R and S-strategies of evolutionary adaptation based on the work of Grime (1979) concerning terrestrial plants. From this C (competitors) equates to 'r' selection, R (ruderals-

disturbance-tolerant) to 'w' selection and S (stress tolerant) to 'K' selection. All algae are not exclusively in one or the other grouping but may show a number of adaptive features leading to a gradual transition between categories. Through the use of these, or similar sets of adaptive categories, phytoplankton species assemblages found at different times of the year have been explained in terms of their different adaptive traits (Sommer, 1981; Reynolds, 1984b, 1988a); this approach can go some way to explaining why certain species bloom under certain conditions.

1.2.2 Phytoplankton

Although the general trend in eutrophic waters is towards increased algal biomass as favourable conditions develop, the actual species composition and their relative biomasses can fluctuate in a series of successions depending on the conditions.

1.2.2.1 Stratified lakes

The stratification of deep lakes into the epilimnion and hypolimnion can play a major role in the phytoplankton ecology of a lake by providing conditions that act to control phytoplankton biomass and species composition.

For growth, phytoplankton need to be in the upper layers of the water column due to their reliance on light and their need for the greater turbulence of the upper layer to keep the cells in suspension. In this situation nutrients can become depleted in the epilimnion, while a rich pool of nutrients may lie in the hypolimnion separated, and therefore largely unavailable to the algae, until stratification breaks down. The breakdown of stratification (overturn) usually begins in the autumn as the epilimnion cools and the wind is able to mix both layers of water, releasing nutrients into the whole water column.

As a very general rule, in a stratified lake the periodicity of the phytoplankton starts with the growth of diatoms in the late winter-early spring before the onset of stratification,

depending on the temperature. The diatom population will then crash to be replaced by small chlorophytes and flagellates which in turn may be replaced by cyanobacteria throughout early to late summer. Another bloom of diatoms may occur in the autumn if mixing of the lake takes place before temperatures become too low at the onset of winter (Round, 1984; Reynolds, 1984a). This is a very general rule and different species may make up the assemblages in different lakes depending on the conditions and levels of trophic. The presence and size of the initial inoculum of a particular species is also very important as to when and how quickly it can grow (Reynolds, 1984b; Lund 1949). Reasons for these successional changes are discussed below.

● Spring

The size and structure of the spring bloom is highly dependent on the physical structure of the water column and hence related to the climate from year to year (Harris, 1986). The diatom bloom occurs as days start to become longer and insolation increases, provided the mixed depth is large enough to keep the cells in suspension since diatoms are prone to sedimentation (Reynolds, 1984b; Harris, 1986).

Species composition and population size is related to the growth rate and initial inoculum size. *Asterionella formosa* Hassall has been shown to be present in the water column throughout the year in lake Windermere, U.K. (Lund, 1949) and it is this ability to maintain a relatively high inoculum throughout the whole year, compared to other species, which allows it to attain a greater biomass in spring. *Fragilaria crotonensis* Kitton, for example, has a lower initial inoculum than *A. formosa* and, therefore, never reaches the population numbers of *A. formosa* (Lund, 1964). *A. formosa* is also able to grow faster than other diatom species such as *Tabellaria flocculosa* (Roth) Kuetz and therefore attains a higher biomass earlier in the year. Both *A. formosa* and *T. flocculosa* can cause problems in water works by blocking filters and producing odours in potable water (Palmer, 1980),

hence, they can be regarded as nuisance algae if they attain large enough numbers in the early part of the year.

The termination of the spring diatom bloom can be brought about by a number of factors such as silicon depletion in the water column (Round, 1984; Reynolds, 1984a) and grazing (Harris, 1986; Reynolds, 1984a). At the onset of thermal stratification and, therefore, a reduction in the mixing depth, diatoms can develop symptoms of photoinhibition and their sinking rates increase (Reynolds, 1984a, 1984b). Under these circumstances diatoms quickly sediment out of the epilimnion and the euphotic zone causing a dramatic decrease in cell numbers.

● Summer

Once stratification has begun and the spring diatom bloom has started to collapse the first species to grow are often small flagellates and/or chlorophytes (Harris, 1986). These C-('r') strategists are generally small and have high energy requirements coupled to high rates of growth, thus requiring longer days and a shallower mixed layer in the euphotic zone. *Scenedesmus* spp. and *Cryptomonas* spp., which can bloom in this period, are known to cause taste and odour problems in potable water supplies (Palmer, 1980). During this period, nutrient depletion and self-shading can act to depress algal numbers, although some flagellates are able to adjust their position in the water column to obtain favourable light, temperature and nutrient conditions (Sommer, 1981 and references therein). Once a certain threshold population of algae is reached, zooplankton such as *Daphnia* spp. are able to increase their population enormously and this may, in turn, have a dramatic effect on algal biomass and community composition, perhaps even preventing nuisance blooms (Harris, 1986).

Grazing of phytoplankton can be important in certain situations and can go some way to changing species composition. Food selection in *Daphnia* appears to be based on

size (Reynolds, 1984b; Sommer, 1981; Porter, 1973) with phytoplankton larger than about $10^4 \mu\text{m}^3$ being infrequently grazed, although the shape of the cell will play a role (Sommer, 1981). Size selection therefore favours larger algal cells and colonies, with smaller cells becoming relatively less abundant. Grazing is just one of the selection pressures which appears to favour the growth of certain cyanobacteria and colonial species over that of the smaller species which are prevalent in the early part of stratification.

Cyanobacteria

During the summer, as stratification continues, the number of bloom-forming cyanobacteria gradually increases. The filamentous form can go some way to reducing losses through grazing. Species such as *Anabaena flos-aquae* Brébisson ex Bornet & Flahault and *Aphanizomenon flos-aquae* Ralfs ex Bornet & Flahault are able to maintain growth at relatively lower light levels than those species with C-strategy traits, hence, they can survive when self-shading has started to reduce the numbers of other species. Many cyanobacteria are also able to withstand the high pH and low CO₂ concentrations which may occur in the epilimnion at this time, caused by the large amount of algal photosynthesis being carried out by other species (Talling, 1976). The presence of heterocysts allows them to fix nitrogen, thus conferring a competitive advantage if nitrogen levels become low in the water. Most planktonic cyanobacteria contain gas vacuoles allowing them to maintain their station in the water column, but they can also act to cause cyanobacteria to float to the surface and form surface scums if certain conditions arise.

Gas vacuoles are made up of numerous hollow cylinders called gas vesicles. They have rigid proteinaceous walls which are permeable to gases but their hydrophobic inner surface prevents the influx of water (Walsby, 1978). It has been shown that there is an inverse relationship between the amount of light received and gas vacuolation in the cells (Reynolds & Walsby, 1975; Fay, 1983). Cells which are deeper in the water column, and hence receiving lower amounts of irradiance, increase the amount of vacuolation through passive gas diffusion into gas vesicles. The more gas that diffuses into the cells the more

buoyant they become and thus they rise up through the water column. As light levels increase, so does the turgor pressure in the cell, causing collapse of the gas vesicles and subsequent sinking of the cell. The rise in turgor pressure is partly due to the increased production of photosynthate in the cell and partly due to light-stimulated uptake of potassium ions which causes an osmotic influx of water (Fay, 1983). Under normal circumstances these two mechanisms operate to regulate buoyancy in the cell, with the increase in turgor pressure preventing the organism from rising too high in the water column and being exposed to photoinhibitory amounts of insolation.

Normally, turbulence in the epilimnion ensures that cyanobacteria become distributed throughout the mixed layer. The average exposure to light means that they do not produce excess photosynthate and therefore, on average, the cells are neutrally buoyant (Reynolds & Walsby, 1975). Problems occur if calm conditions are encountered in the water body. In this situation, in the absence of turbulence, the cells will start to rise up through the water column to form a dense surface bloom. Blooms are likely to occur if the cells pass from light-limiting to light-inhibiting conditions before they have time to react, or if the population rises during the night (Reynolds & Walsby, 1975). In both instances photo-oxidation and cell lysis is likely to occur unless turbulence can force the cyanobacterial population back into the water column (Fay, 1983).

Towards the end of summer the colonial cyanobacterium *Microcystis aeruginosa* Kütz. emend. Elenkin is often the dominant alga and can form blooms via the buoyancy mechanisms mentioned previously. Reynolds (1984a) classes this species as strongly 'k' selected (S-strategist from Reynolds, 1988a), building up its numbers throughout the summer period. Growth is initiated from colonies which have overwintered on the bottom sediments (Reynolds *et al.*, 1981). Low irradiance, oxygen concentration and redox potential appear to be essential to initiate growth, hence the low oxygen concentrations of the hypolimnion provide an ideal habitat and stimulus for *Microcystis* growth. The growth of *M. aeruginosa* is accompanied by low loss rates since the buoyancy mechanism can

keep the colonies in suspension and the colonial form prevents grazing of most colonies exceeding 20-60 μm in diameter (Reynolds *et al.*, 1981).

Another group of algae suited to the conditions of late summer/autumn are the desmids. This may be related to their lack of motility and relatively large size meaning they are suited to deeper mixed layers where the cells can be kept in suspension. They are also more dependent on the higher temperatures of this period, rather than those found in the spring period (Canter & Lund, 1966). Desmids generally favour oligotrophic waters, but some species such as *Staurastrum pingue* are known to form blooms under eutrophic conditions (Brook, 1981), possibly introducing grassy odours into potable water (Palmer, 1980).

● Autumn

As autumn approaches, temperature and the amount of light entering the system decrease. Cooling of the surface water causes it to sink, to be replaced by warmer water from below, thus increasing the depth of the epilimnion and therefore the mixed layer. Eventually wind mixing and convectional cooling isothermally mix the water reintroducing, nutrients back into surface waters (Round, 1984). The increase in depth of the mixed layer presents increasingly unfavourable conditions for the summer species and is better suited to the diatoms. Generally the species which were abundant in the early-spring period are best able to dominate in autumn, since they provide inocula from residual surviving populations in the water column or from the sediments (Reynolds, 1984a). The appearance and size of the autumn diatom bloom depends on the weather conditions at the time, but common species occurring in eutrophic lakes include *Fragilaria crotonensis*, *Aulacoseira granulata* (Ehrenberg) Simonsen and *Asterionella formosa*, all of which are known to clog water extraction filter screens and introduce odours into potable water (Palmer, 1980). Other species such as *Microcystis aeruginosa* may also survive well into the autumn period depending on local conditions (Fay, 1983; Reynolds, 1984a).

1.2.2.2 Shallow water bodies (non-stratified)

In shallow lakes thermal stratification may not occur for long periods, so the mixed depth can be considered as equal to the depth of the lake (Scheffer *et al.*, 1997). Under eutrophic conditions shallow lakes often tend to be turbid since mixing continuously introduces sediment into the water column and prevents settling of particulate organic matter which is either produced (e.g. algae) or introduced into the water column (Moss *et al.*, 1996a).

Shallow, turbid lakes often have a close to continuous growth season dominance of species of *Oscillatoria*, particularly *O. redekei* van Goor and *O. agardhii* (Jurgens) Gomont (Gibson & Smith, 1982; Reynolds, 1994a; Scheffer *et al.*, 1997). This dominance comes about largely due to their high adaptive capability to exist at low levels of insolation (Reynolds, 1994a). The cyanobacteria can also promote such conditions since they cause higher turbidity per unit of phosphorus than other algae (Scheffer *et al.*, 1997). The almost neutral buoyancy of these cyanobacteria allows them to remain in suspension and their filamentous form makes them largely inedible to zooplankton (Foy *et al.*, 1976; Reynolds 1988a).

A large influence in shallow lakes of less than about four metres in depth may be the growth of vascular aquatic plants (macrophytes). The interaction between algae and macrophytes is complex and eutrophication can switch an ecosystem from one with clear water which is dominated by macrophytes, to one with turbid water which is dominated by phytoplankton. This interaction is discussed more fully in chapter five.

Morphometric and hydrological processes also play their part in influencing the algal flora. Shallow lakes or ponds with a large surface area to volume ratio allow nutrients to become concentrated into a relatively small volume of water, which can lead to hypertrophy. Under these conditions the water is often dominated by fast-growing Chlorococcales and Volvocales (Reynolds & Walsby, 1975). If high nutrient loading is

achieved by high rates of inflow it will be those species which can grow faster than the water retention time which will dominate.

1.2.2.3 Rivers

Compared to the plankton of lakes there are relatively few species that can achieve abundance in rivers (Reynolds, 1994a). The nature of the riverine system means that species which reproduce quickly are selected for, since slower growing species would be washed out of the system before achieving any degree of abundance. Algal biomass increases from the headwaters towards the lower reaches (Reynolds, 1995) as the slope decreases and more nutrients become available from the surrounding catchment. There is also a change in species composition from Chlorophytes (mainly members of the Chlorococcales) in upstream areas towards an increasing biomass of diatoms in the lower reaches. Reynolds (1994b) suggested that this increase was due to the diatoms' greater ability to intercept light by virtue of accessory pigmentation. This makes diatoms better adapted than chlorophytes to the intermittent photoperiods in the well-mixed, turbid environment of the lower reaches of a river. The relative composition of biomass throughout the year is similar to that of lakes in many instances, with a higher proportion of green algae occurring during the summer (Whitton, 1975) and diatoms showing markedly higher growth in spring and autumn.

If the speed of a river and the numbers of phytoplankton sampled are taken into account there is a paradox in that there appear to be more cells than could possibly have grown in the time taken to travel the whole of the river channel (Reynolds, 1988b, 1995, 1996). That potomoplankton exist in the numbers seen is due to the spatial variations in turbulence across a river channel (Reynolds, 1995, 1996). A river channel does not have a constant flow within it but it is patchy consisting of pools, riffles and rapids (Reynolds, 1994a, 1995, 1996) which vary depending on weather-related changes in the volume of water in the channel. This variability means dead zones can be formed where algae are retained and separated from the flow of the main channel. In these dead zones algae are

able to reproduce in greater numbers than in the main channel, but fluid exchange will still occur between the two water masses 'seeding' the main channel with extra algae. In general, the weaker the slope the greater the number of dead zones or retentivity of the channel (Reynolds, 1995). Thus, algal biomass and therefore the possibility of nuisance algae is increased in the lower reaches of a river where, for example, blooms of *Euglena*, *Microcystis* and *Aphanizomenon* can occur (Prescott, 1969). In the faster-flowing parts of a river it is the periphyton of attached filamentous algae which are likely to grow to nuisance proportions.

1.2.3 Filamentous algae

Filamentous algae may be attached or free-floating, although attached forms often become detached during the growing season, especially after flooding (Whitton, 1971). Whitton (1971) suggested that filamentous forms are more likely to grow to nuisance proportions at intermediate levels of 20-200 $\mu\text{g l}^{-1}$ of dissolved inorganic phosphorus since, at very high nutrient levels, the filamentous forms are often out-competed by planktonic algae, at least in small lakes and ponds.

1.2.3.1 Free-floating filamentous algae

The wholly free-floating forms such as *Spirogyra* and *Hydrodictyon* are generally restricted to static waters such as ponds and sheltered littoral areas of lakes (Lembi *et al.*, 1988), although there are exceptions (*Hydrodictyon reticulatum* in Loe pool, U.K., for example (Flory & Hawley, 1994; John *et al.*, 1998)). The algae are able to begin growth in the spring period on the bottom sediments from over-wintering spores, zygotes or filaments. The entrapment of oxygen bubbles within the algal mass allows the algae to float to the water's surface to form nuisance mats.

In temperate areas, members of the Zygnemataceae, such as *Spirogyra* spp., are often first to grow in spring. *Cladophora glomerata* often then becomes dominant when it

detaches from the substrate (Lembi *et al.*, 1988; Dodds & Gudder, 1992), while species of *Oedogonium* may come to dominate the flora from late summer to early autumn.

1.2.3.2 Attached filamentous algae

Attached forms, by their very nature, are more abundant in littoral areas of lakes and can become dominant in enriched riverine systems, where taxa such as *Cladophora*, *Stigeoclonium*, *Ulothrix* and *Rhizoclonium* are common (Palmer, 1980). *Cladophora glomerata* is able to withstand the shear stresses of benthic areas (Dodds & Gudder, 1992) and is resistant to grazing (Dodds, 1991; Dodds & Gudder, 1992), so it is very common in nutrient-enriched rivers and littoral areas of lakes.

A common genus which can cause problems especially in drainage ditches is *Vaucheria* (Whitton, 1971; Dowidar & Robson, 1972) (see Chapter 5). *Vaucheria* can form floating mats, but is frequently implicated in the siltation of ditches as it can grow with part of its thallus submerged in the sediment. Its growth habit allows it to extract nutrients from the sediment, even when nutrients in the surrounding water may be low (Whitton, 1971). Its attached growth form also makes it very difficult to remove physically and as such it is considered a major nuisance to land drainage engineers (G. Cave, pers. comm.).

Under favourable growth conditions benthic algal populations often develop profusely when stream velocities are seasonally reduced. These populations commonly become physically unstable on the substrate so that when current velocity increases large numbers of filaments may be released into the water to accumulate and create a nuisance.

1.2.4 Conclusion: the causes of bloom formation

The accumulation of algal biomass is brought about by the maximization of factors favouring growth and the minimization of loss factors (Harris, 1986). In the case of algal blooms, the addition of excess nutrients allows growth to override loss factors and biomass can increase to excessively high levels. However, not all the species in an algal community

grow to nuisance proportions. Individual growth characteristics and the biology of an organism interacting with abiotic and biotic factors of the surrounding environment will determine the eventual biomass a species can attain. It follows that those species with attributes which make them best able to exploit excess nutrients, or conditions caused as a direct result of excess nutrients, will be most likely to grow to nuisance proportions.

1.3 Control of algal blooms

The prevention of excessive agricultural run-off by the use of buffer zones around a water body, or the removal of phosphorus and nitrogen from sewage effluent before discharge, will clearly stop nutrients getting into a water system in the first place and prevent excessive algal growths from ever happening. However, over many years of nutrient loading, nutrients such as phosphorus bind to aquatic sediments so that, even if all nutrient inputs could be stopped immediately, nutrients will still be available for algal growth via their gradual release from sediments. Methods that have been employed to try and stop the release of phosphorus from sediments include physically sealing the sediment from the water with plastic sheeting or fly ash, chemically sealing the sediment with iron sulphate to form a phosphorus-binding cover at the sediment surface and even removing a certain amount of the sediment (Mason 1991; Moss *et al.*, 1996a). These methods have met with varying degrees of success and some are regarded as environmentally damaging since damage to benthic fauna is inevitable.

More effective control methods rely on the use of chemicals to inhibit algal and cyanobacterial growth, but these are seen as increasingly controversial with the increased public awareness of ecological matters. Most aquatic herbicides are non-selective, removing higher plants as well. This may reverse the desired effect since the removal of macrophytes will decrease competition, possibly leading to an increase in algal growth. Certain species of algae also appear to have become resistant to some herbicides; for example, Gibson & Barrett (1989) showed that the filamentous alga *Vaucheria dichotoma* (L.) Martius developed resistance to the herbicide terbutryn in drainage channels. Copper

sulphate has been used as a selective algicide but is now banned in many countries because of its toxicity to freshwater fauna and concern over the fate of copper residues which may accumulate in sediments and in the food chain.

The physical removal of filamentous algae from water is another option to reduce the algal biomass present, but this requires specialized equipment and can be highly labour intensive. Physical removal is only a temporary measure and the removal of macrophytes along with algae may exacerbate the problem by reducing the competition between macrophytes and algae. Disposal of the algae, after removal, can also present a significant problem, especially if the biomass is considerable.

Biomanipulation has been widely employed in lake restoration schemes as a means to reduce excessive algal growths (e.g. Galanti *et al.*, 1990; Meijer *et al.*, 1990; Moss *et al.*, 1996b) (see Chapter 5). For example, the removal of all fish from a waterbody, or the introduction of piscivorous fish, will reduce the numbers of fish which feed on algal grazers such as *Daphnia* spp., thus allowing an increase in the numbers of algal grazers. The increase in algal grazers produces a concomitant reduction in algal biomass. In some specialized situations grass carp can be deployed to eat filamentous algae (Eaton *et al.*, 1992), although this is not an option for larger water bodies or those stocked for a particular type of fishing.

For all methods of algal control there is generally at least one factor which causes a problem whether it be cost, ecological concerns or variable success in different situations. It is always advantageous, therefore, to introduce new methods of algal control which will complement or replace existing methods.

1.3.1 Use of barley straw for algal control

In 1979-80 algal growth was reported to be reduced in the presence of rotting straw or hay (reported in Wingfield *et al.*, 1985). Wingfield *et al.* (1985) reported that a reduction in

phosphate levels accompanied straw decomposition and proposed that the microbial decomposition of straw could cause a reduction in inorganic nutrient supply in the surrounding water. Further studies by Welch *et al.* (1990) in the Chesterfield canal and Gibson *et al.* (1990) in the laboratory showed that rotted barley straw (*Hordeum vulgare* L.) was indeed inhibitory, but they concluded that the inhibitory action was not due to reduced nutrient levels in the water but to the production of algal inhibitor(s). Pillinger *et al.* (1992) showed that the inhibitory component was most likely to come from the straw itself mediated by, but not from, the associated mycoflora, although the production of algal antibiotics by bacteria or actinomycetes was not ruled out. In the laboratory, a number of green algae and cyanobacteria have been shown to be inhibited by decomposed barley straw (Gibson *et al.*, 1990; Newman & Barrett, 1993) but with doses of up to 10^4 g dry mass m^{-3} of growth medium. In the field, control can be achieved with as little as 3-50 g m^{-3} (Barrett *et al.*, 1996; Ridge & Pillinger, 1996; Ridge *et al.*, 1995). Barley straw is now in widespread use as a method of algal control since it is relatively cheap and, up to the present, no adverse ecological effects have been reported. The growth of macrophytes and animal life appears unaffected (Everall & Lees, 1996) and barley straw has been used in potable water supplies where no unusual amounts of organic chemicals monitored by the water industry have been found (Barrett *et al.*, 1996).

1.3.1.1 Overview of the algal inhibitor(s)

As well as barley straw (Welch *et al.*, 1990; Gibson *et al.*, 1990), brown-rotted wood (Pillinger *et al.*, 1995) and oak leaf litter (*Quercus robur* L.) (Ridge *et al.*, 1995) have all been shown to be inhibitory towards the growth of cyanobacteria and algae when decomposed aerobically in water. The inhibitory potential towards algae has been shown to last for many months in the case of oak leaves (Ridge *et al.*, 1995) and barley straw (Pillinger, 1993). However, the active component(s) can be unstable (Welch *et al.*, 1990) and can be rendered inactive by adsorption onto sediments and onto the algae themselves (Barrett, 1994); thus, the inhibitor(s) need to be continually released from plant litter to produce inhibition in most cases. Pillinger *et al.* (1994) concluded that the inhibitor(s)

appears to be some form of oxidized polyphenolic compound derived from lignin which is solubilized from the plant material. The structure and composition of lignin may play a part in the potency of the inhibitor(s). Pillinger *et al.* (1995) suggested that the ratio of syringyl units to guaiacyl units in lignin of rotted wood samples would confer different amounts of inhibition since a high ratio of syringyl units shows a greater potency than a lower ratio. This may be related to the greater methoxy substitution of syringyl units and their greater ease of oxidation. Pillinger (1993) showed that aerobic conditions were of paramount importance for inhibition to occur.

1.4 Natural dissolved organic carbon in freshwater

In freshwaters there are three classes of organic carbon: coarse particulate organic carbon (CPOC), fine particulate organic carbon (FPOC) and dissolved organic carbon (DOC), all of which are considered analogous to organic matter (Thurman, 1985). Thurman (1985) defines DOC as the organic carbon passing through a 0.45 μm filter, with rivers and lakes generally containing about 2-10 mg l^{-1} DOC. DOC is heterogeneous, with some compounds being utilized by bacteria and other heterotrophs while other components, recalcitrant to microbial action, accumulate in the system and play a role in the formation of humic substances (although there is now evidence that these so-called 'recalcitrant' substances can sometimes be utilized by bacteria (Bushaw *et al.*, 1996)). In natural ecosystems DOC comes from a number of sources such as bacterial activity in sediments, rainfall run-off through soil and from natural plant litter inputs. Organic matter from plants is more readily available to micro-organisms and, therefore, produces a wider range of chemicals than that from soil, which has been decomposed for longer and as such is more recalcitrant. Generally, primary production rates and plant litter decomposition rates control the amount of DOC in a water body, with the rate of formation of DOC and its subsequent fate being important in the context of algal inhibition.

1.4.1 Effects of dissolved organic carbon/matter on algal growth

Saunders (1957) proposed that DOC can exert its effects on organisms in four basic ways:

(a) It may supply compounds which can be utilized as energy sources by algae and cyanobacteria or it may contain one or more of the basic elements essential for growth and functioning

It has been known for a long time that certain algae can grow completely heterotrophically or photoheterotrophically by utilizing organic substrates (Saunders, 1957; Droop, 1974 and references therein). In this case, utilization of DOC stimulates growth or allows growth under adverse conditions, such as lowered light levels.

(b) DOC may supply accessory growth factors which can stimulate algal growth.

It is widely accepted that some algae require accessory growth factors due to the necessity of adding yeast extracts or soil to algal cultures in the laboratory to obtain growth. It is thought that the addition of these materials provides small amounts of vitamins for the growing algae (Prescott, 1969). Other unidentified compounds, obtained from soil or yeast, may play a role in some species which do not grow in the laboratory even with the addition of vitamins to a mineral growth medium

(c) It may take the form of toxic/stimulatory substances which inhibit or stimulate the growth of organisms

If in large enough quantities, simple phenolic acids may play a role in regulating algal density because of their demonstrated plant growth regulatory activity (Larson, 1978) and general toxicity (Newman & Barrett, 1993; Everall & Lees, 1997). Humic substances, as known surfacants, may affect algal cell membranes or directly affect the intra and/or extracellular enzymes of algae (Perdue & Gjessing, 1989; Wetzel, 1992). Binding and complexing can take place with a number of enzymes such as phosphatases, glucosidases and aminopeptidases which can affect the growth of algae (Boavida & Wetzel, 1998).

(d) *It may have indirect effects such as complexing with trace metals*

Chelation and complexation of trace metals with organic compounds can have various consequences. The organic chelate may lower the concentration of trace metals to below the level required for growth of an organism (Saunders, 1957; Wetzel, 1992; Thomas, 1997). Conversely, if there is an excess of a trace metal, making it toxic, chelation may reduce the metal concentration to a non-toxic level. Chelation can also maintain a particular element in a soluble state when normally that element would precipitate out of solution.

Between 30 and 60% of all DOC in natural waters is made up of humic substances. These are organic acids of between 500-10000 molecular mass from which all easily available energy has been extracted (Bushaw *et al.*, 1996). Humic substances and phenolic acids can have various effects in natural freshwaters (Saunders, 1957; Larson, 1978; Thomas, 1997; Wetzel, 1992) through processes mentioned above, although Pillinger (1993) and Pillinger *et al.* (1994) suggested that phenolic acids and simple phenolic compounds were not present in high enough concentrations to account for the inhibition of algal growth obtained with barley straw. What is of interest in the present study of algal inhibition is the polyphenolic fraction of plant litter released from solubilized lignin over a long period of time; not the simple organic acids leached from plant material over a few days or weeks, but the precursory chemicals which, through further degradation and oxidation, form recalcitrant humic acids. It is these oxidized polyphenols that appear to inhibit algal growth over longer periods of time and are more likely to have effects in natural freshwaters, especially where litter inputs are large or concentrated in debris dams (Bilby, 1981). However, Everall & Lees (1997) hypothesized that algal control may have been exerted in a Derbyshire reservoir via a 'cocktail' of organic chemicals including organic acids as well as larger polymeric fractions.

1.5 Aims of the study

The major aims of this study were two-fold:

(a) Determine the sensitivity of a range of algal and cyanobacterial species to plant litter in laboratory studies

It had been suggested that diatoms appear to be resistant to plant litter-derived inhibitor(s) since one species was found to grow in tanks of rotting barley straw (reported in Ridge *et al.*, 1995). This and other work (Newman & Barrett, 1993; Gibson *et al.*, 1990), where cyanobacteria and green algae were tested, suggested that not all algae were susceptible or inhibited to the same degree. Comparisons of the differing sensitivities of a wide range of taxa may give clues as to the mode of action of the inhibitor(s) from comparisons of the underlying physiology and biochemistry of the species' in question. Also, comparisons of sensitivity may have relevance for barley straw application in the field, since barley straw may selectively inhibit certain species and allow previously non-nuisance species to grow to nuisance proportions.

The species to be assayed with the decomposing plant litter were chosen because of their morphological and/or taxonomic characteristics or for their known ability to grow to nuisance proportions, the details of which are explained more fully in Chapter three.

(b) Assess any changes which occur in the field when using barley straw to suppress algal growth

By surveying sites where barley straw was being used it was hoped that any change in the relationship between macrophytes and algae (see Chapter 5) or biomass and species composition could be determined. The presence or absence of particular species' may also help to verify the laboratory studies of (a) and vice-versa. Field studies could also help to determine whether algae eventually become resistant to barley straw in the long term.

Chapter 2

Methods for laboratory studies

A list of suppliers from whom experimental equipment and supplies were obtained is shown in Appendix 1. Not all of the measurement units used in this thesis adhere to the *Système Internationale d'Unités* (SI units). I have chosen units which reflect those in common use in the general literature (e.g. days instead of seconds) and in literature pertaining to decomposed plant litter. Appendix 2 lists the units used and their equivalent SI units.

Initial investigations were carried out by assaying algal growth with different amounts of decomposed oak leaves or barley straw under defined laboratory conditions.

2.1 Culture conditions

2.1.1 Light and temperature

Algae were grown under continuous light of $120 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation (PAR) supplied by cool white fluorescent tubes, lit from above, in a growth cabinet (SANYO-Gallenkamp). Cyanobacteria were grown in the same growth cabinet but under a reduced light intensity of $60 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR obtained by placing one layer of 'cheese cloth' over the culture vessels. The use of a light:dark regime was considered unnecessary since previous work had shown straw to inhibit algae under continuous light (Pillinger, 1993; Pillinger *et al.*, 1994). Continuous light was used since it produces faster growth and quicker results (Nyholm & Kallqvist, 1989). Possible shading of algae by the plant litter was considered negligible with the amounts used in these assays since previous work (results not shown), and work using higher masses of barley straw (Gibson *et al.*, 1990), showed no shading effect.

All species were grown at a temperature of $20^{\circ}\text{C}\pm 1^{\circ}\text{C}$ which, although not optimum for any particular species, allowed a wide range of species to be grown simultaneously.

2.1.2 pH

Whether pH should be controlled in algal toxicity tests or allowed to drift has been widely debated (Nyholm & Kallqvist, 1989). In nature large diurnal variations in pH may occur in productive and/or weakly buffered systems due to the effects of photosynthesis, which can change the speciation of toxic chemicals (Saarikoski & Vilukelsa, 1981). It could be argued therefore that toxicity tests should be carried out without pH control, so that during the test, the algae will be exposed to the toxicant at a range of pH values. However, the results from such tests will be difficult to reproduce and since toxicity testing using plant litter often gives variable results, largely due to the biologically mediated nature of inhibitor release, it was thought necessary to control the pH. Culture flasks were shaken twice daily to try and ensure the mass transfer conditions for carbon dioxide into the medium were adequate for algal growth and to prevent excessive uptake and use of bicarbonate by some species, which could increase the pH. Shaking also kept the algae in suspension and prevented sinking and self-shading. Continuous shaking or aeration of cultures would have been preferable (Nyholm & Kallqvist, 1989) but facilities were not available for this. One particular fixed pH was not used due to the wide range of species to be tested and the different growth media which were used, although the pH was kept in as narrow a range as possible (7.2-8.2) to reduce any possible pH effects. A basic pH was used to ensure favourable conditions for the autoxidation of phenolic compounds solubilized from the plant litter (Pillinger *et al.*, 1994).

The buffer used in the assays was 4-(2-hydroxyethyl) piperazine-1-ethane sulphonic acid sodium salt or HEPES (ICN). It has a buffering range of 6.8-8.2, does not require an enriched carbon dioxide atmosphere to maintain the correct pH and does not bind to divalent cations in growth media (ICN, 1997), thus preventing any interaction with nutrient supply to the test organism. HEPES was on the upper limit for buffering the JM1

medium (see Table 2.1) but the pH was found to vary by not more than 0.2 units during assays (results not shown).

2.1.3 Exponential growth

It has been suggested that toxicity tests should preferably be restricted to the initial period of exponential growth (Nyholm & Kallqvist, 1989). This will prevent erroneous results being obtained if control cultures become nutrient limited. A comparison between toxicant-treated cultures and nutrient-limited control cultures will show relatively less inhibition in the toxicant-treated cultures due to the reduced biomass of the control cultures at a given time. Also, during the course of a bioassay the toxicity of the chemical(s) in question may be reduced by various mechanisms, in particular by adsorption onto the algal cells. For these reasons, growth over time was measured for some of the test species in the early part of the study so that the onset of exponential growth could be found.

Experience in the later stages of the study meant that growth curves were not plotted for all species since it was possible to assess visually when cultures were exponentially growing. Quantitative and qualitative study (colour of flasks) also showed that it did not affect the susceptibility/resistance of the algae whether they were sampled in the early or late-exponential phase. If the algae were susceptible to the plant litter the inhibition of growth remained throughout a long time course, even when the controls had become nutrient limited. Throughout the whole study only one species (*Scenedesmus quadricauda*) was found to recover cell numbers after an initial inhibition of growth (see section 3.3.2)

2.1.4 Stock cultures

Cultures were maintained through continuous sub-culture by inoculation of 1 ml of an exponentially growing culture into a flask containing 50 ml of the relevant medium and plugged with non-absorbent cotton wool. The stock cultures were shaken twice daily. All media were previously autoclaved (Dixons surgical instruments) for 15 minutes at 121°C

and a pressure of 15 psi, left to cool for at least six hours at ambient temperature and inoculated aseptically in a laminar flow cabinet (MDH, microflow). No attempt was made to use or keep the unialgal cultures axenic.

2.1.5 Growth media

Most of the bioassays were carried out over a short period of time so it was thought unlikely that algae would experience nutrient-limiting conditions. For those assays carried out over longer periods of time the growth curve of the control cultures over time showed when the organisms were becoming resource-limited. Algae and cyanobacteria were grown in JM1 medium where possible (Table 2.1) but other media were used when necessary to obtain adequate growth of the cultures (see Tables 2.2-2.4).

Unless otherwise stated all stocks of chemicals were kept dark at room temperature. All media were buffered with 20 mM HEPES adjusted with 5 M NaOH or 1 M HCl to obtain the required pH. All chemicals were supplied by BDH-Merck apart from cyanocobalamine, thiamine and biotin which were obtained from Sigma. Solutions were made with glass distilled water.

JM1

Stock number	Compound	Concentration of stock solution (mM) ¹	Concentration in medium (μM)
1	Ca(NO ₃) ₂ .4H ₂ O	85 (20)	85
2	KH ₂ PO ₄	176 (24)	176
3	MgSO ₄ .7H ₂ O	203 (50)	203
4	NaNO ₃	941 (80)	4705
5	EDTA FeNa	6 (2.25)	6
	EDTA Na ₂	7 (2.25)	7
6	H ₃ BO ₃	40 (2.48)	40
	MnCl ₂ .4H ₂ O	7 (1.39)	7
	(NH ₄) ₆ Mo ₇ O ₂₄ .4H ₂ O	0.8 (1)	0.8
7	NaHCO ₃	189 (15.9)	189
8	Cyanocobalamine	0.03 (0.04)	0.03
	Thiamine HCl	0.2 (0.04)	0.2
	Biotin	0.2 (0.04)	0.2
9	K ₂ HPO ₄ .3H ₂ O	101 (23)	101

Table 2.1 Jaworski's medium (JM) (Tompkins *et al.*, 1995) modified by the Aquatic Weeds Research Unit (see Pillinger, 1993) and adjusted to pH 8.2. Stock 8 kept refrigerated at 4°C. 1 ml of each stock solution was used in each litre of growth medium, apart from stock 4 which used 5 ml of stock solution. ¹Values in parentheses show the mass of the compound used in the stock solution (g l⁻¹).

DM

Stock number	Compound	Concentration of stock solution (mM) ¹	Concentration in medium (μM)
1	Ca(NO ₃) ₂ ·4H ₂ O	85 (20)	85
2	KH ₂ PO ₄	91 (12.4)	91
3	MgSO ₄ ·7H ₂ O	101 (25)	101
4	NaHCO ₃	189 (15.9)	189
5	EDTA FeNa	6 (2.25)	6
	EDTA Na ₂	7 (2.25)	7
6	H ₃ BO ₃	40 (2.48)	40
	MnCl ₂ ·4H ₂ O	7 (1.39)	7
	(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	0.8 (1)	0.8
7	Cyanocobalamine	0.03 (0.04)	0.03
	Thiamine HCl	0.2 (0.04)	0.2
	Biotin	0.2 (0.04)	0.2
8	NaSiO ₃ ·9H ₂ O	201 (57)	201

Table 2.2 Diatom medium after Tompkins *et al.* (1995) and adjusted to pH 8.0. Stock 7 kept refrigerated at 4°C. 1 ml of each stock solution was used in each litre of growth medium. ¹Values in parentheses show the mass of the compound used in the stock solution (g l⁻¹).

'WC'

Stock number	Compound	Concentration of stock solution (mM) ¹	Concentration in medium (μM)
1	CaCl ₂ .2H ₂ O	250 (36.8)	250
2	MgSO ₄ .7H ₂ O	150 (37)	150
3	NaHCO ₃	150 (12.6)	150
4	K ₂ HPO ₄	50 (8.7)	50
5	NaNO ₃	1000 (85)	1000
6	NaSiO ₃ .9H ₂ O	100 (28.4)	100
7	EDTA Na ₂	13 (4.4)	13
	FeCl ₃ .6H ₂ O	12 (3.2)	12
	CuSO ₄ .5H ₂ O	0.04 (0.01)	0.04
	ZnSO ₄ .7H ₂ O	0.08 (0.02)	0.08
	CoCl ₂ .4H ₂ O	0.04 (0.01)	0.04
	MnCl ₂ .4H ₂ O	0.91 (0.18)	0.91
	Na ₂ MoO ₄ .2H ₂ O	0.025 (0.006)	0.025
8	Cyanocobalamine	4 x 10 ⁻⁴ (5 x 10 ⁻⁴)	4 x 10 ⁻⁷
	Thiamine HCl	0.3 (0.1)	0.3
	Biotin	0.002 (5 x 10 ⁻⁴)	0.002

Table 2.3 'WC' medium after Guillard & Lorenzen (1972) but modified by using HEPES buffer instead of Tris buffer and adjusted to pH 7.2. Stock 8 kept refrigerated at 4°C. 1 ml of each stock solution was used in each litre of growth medium. ¹Values in parentheses show the mass of the compound used in the stock solution (g l⁻¹).

compound		per litre
Stock	CaCl ₂ stock solution	
	CaCl ₂	9 mM (1.0 g)
Medium	Sodium acetate trihydrate	1.0 g
	* 'Lab-Lemco' powder	1.0 g
	* Tryptone	2.0 g
	* Yeast extract	2.0 g
	CaCl ₂ stock solution	9 µM (10 ml)

Table 2.4 *Euglena* medium after Tompkins *et al.* (1995) and adjusted to pH 8.0. The table shows the amount of compound required for one litre of growth medium.

Footnote: * supplied by Unipath (Oxoid) Ltd U.K.

2.2 **Decomposition of the plant litter**

Both barley straw and oak leaf litter were decomposed in aged tap water in the dark at room temperature. Aged tap water was obtained by bubbling water with air for at least 3 days before use (Gibson *et al.*, 1990; Pillinger, 1993). Plant litter decomposition was carried out in glass tanks bubbled vigorously with air via aquarium pumps (Whisper) through 25 ml glass pipettes. Decomposition was carried out in the dark (by covering tanks with black plastic) to prevent the growth of any resistant algae which could have interfered with results, especially when using chlorophyll extraction for biomass estimation (section 2.3.3.2)

2.2.1 Barley straw

Barley straw was obtained from Croydon Hill farm near Cambridge, U.K. and stored dry before use. Straw was added to the water at 2 g l⁻¹ dry mass (Pillinger, 1993; Pillinger *et al.*, 1994) and all straw used in experiments was decomposed for between two and six months.

2.2.2 Oak leaves

Oak leaves were collected from the Open University campus, Milton Keynes, U.K. within four weeks of abscission, sorted to remove twigs and most leaves of other species and stored dry. Leaves collected in 1995 and 1996 were used in all experiments, added to aged tap water at 5.5 g l⁻¹ (Ridge *et al.*, 1995) and aerated under the same conditions as those used for barley straw.

2.3 Bioassays

One bioassay technique initially investigated was the use of agar plates to grow algae (Pillinger *et al.*, 1992; Pillinger, 1993). Although relative sensitivity could not be compared quantitatively using this method, it was hoped that it could provide a means to screen large numbers of species quickly and efficiently. More detailed investigations could then be carried out if any interesting results were obtained.

2.3.1 Agar plates

Initial investigations used the green alga *Chlorella vulgaris* Beijerinck. Agar plates made up with JM1 medium were inoculated with *C. vulgaris* and various treatments of plant litter were placed onto the plates. The treatments included the direct addition of barley straw and oak leaves onto the surface of the plates, or the addition of the water in which the plant litter was degraded into a well made in the agar. The presence or absence of

inhibition zones in the algal lawn surrounding the litter treatment, after several days growth, could be used to determine the response of the species.

After many replications it was decided not to use this type of assay for future work since no inhibition zones were seen in most assays. The results were considered too variable to obtain any useful information and future work concentrated on liquid assays in flasks.

2.3.2 Flask bioassay

Assays were carried out based on the methods of other workers (Gibson *et al.*, 1990; Pillinger, 1993; Newman & Barrett, 1993; Ridge *et al.*, 1995). In all cases 150-ml or 100-ml conical flasks containing 50 ml of the relevant growth medium were used. After the addition of the plant litter the flasks were inoculated with 1 ml of an exponentially growing culture of algae/cyanobacteria and plugged with non-absorbent cotton wool. Tests with *Hydrodictyon reticulatum* were performed slightly differently (see section 3.3.2). The provenance of all the algal cultures which were used are shown in Table 2.5.

Table 2.5 Origin of cultures used during the laboratory investigations.

Footnote: SAG cultures from Sammlung von Algenkulturen at the University of Göttingen, Germany

CCAP cultures from Culture collection of algae and protozoa, Windermere, U.K.

Sciento cultures from Sciento, Manchester, U.K.

Microcystis aeruginosa (AK1) from Professor G. Codd, Dundee University

Stichococcus bacillaris from the Natural History Museum culture collection, isolated from River Towy, Wales

Synechococcus sp. isolated as a contaminant species which took over a *M. aeruginosa* culture after accidental heating to ca. 35 °C.

The very common nuisance filamentous algae *Cladophora glomerata* and *Vaucheria* spp. were not investigated in laboratory assays. *C. glomerata* had previously been shown to be inhibited by decomposing barley straw both in the field (Welch et al., 1990) and the laboratory (Gibson et al., 1990). Attempts were made to grow *C. glomerata* and *Vaucheria dichotoma* in the laboratory and in growth chambers in replicated ponds at the Open University campus. However, no growth, or negligible growth, was obtained in control cultures, which was unsatisfactory for further assays. The susceptibility of *V. dichotoma* was tested in field trials (see Chapter five).

	Species	Origin
Heterokontophyta	Bacillariophyceae	
	Pennales	
	<i>Asterionella formosa</i> Hassall	Sciento (A435)
	<i>Tabellaria flocculosa</i> (Roth.) Kuetz	Loch nam Brac Grid ref. NC1848
	<i>Nitzschia filiformis</i> (W. Smith) Van heurck var. <i>conferta</i> (Richter) Lange-Bertalot	Isolated from tank of decayed oak leaves
Chlorophyta	Zygnematophyceae	
	Desmidiales	
	<i>Closterium ehrenbergii</i> Meneghini ex Ralfs	SAG 134.80
	<i>Staurostrum pingue</i> Teiling	SAG 5.94
	<i>Cosmarium biretrum</i> (de Brébisson) Ralfs	SAG 44.86
	Zygnematales	
	<i>Spirotaenia erythrocephala</i> Itzigsohn	SAG 7.89
	Chlorophyceae	
	Chlorococcales	
	<i>Chlorella vulgaris</i> Beijerinck	CCAP 211/12
	<i>Scenedesmus subspicatus</i> Chodat	CCAP 276/20
	<i>Scenedesmus quadricauda</i> (Turpin) Brébisson (=S. communis Hegewald)	CCAP 276/21 Sciento (A294)
	<i>Hydrodictyon reticulatum</i> (L.) Lagerheim	Sciento (A162)
	<i>Pediastrum boryanum</i> (Turpin) Meneghini	Sciento (A250)
	Klebsormidiophyceae	
	Klebsormidiales	
	<i>Stichococcus bacillaris</i> Nägeli	BM 83092907A
Euglenophyta	Euglenophyceae	
	Euglenales	
	<i>Euglena gracilis</i> Klebs	Sciento (P530)
Eubacteria		
Cyanophyta	Cyanophyceae	
	Nostocales	
	<i>Anabaena flos-aquae</i> Brébisson ex Bornet & Flahault	CCAP 1403/13B
	<i>Anabaena cylindrica</i> Lemmermann	Sciento (A490)
	<i>Aphanizomenon flos-aquae</i> Ralfs ex Bornet & Flahault	CCAP 1401/1
	Oscillatoriales	
	<i>Oscillatoria redekei</i> van Goor	CCAP 1459/29
	<i>Oscillatoria animalis</i> Agardh	Sciento (A565)
	Chroococcales	
	<i>Microcystis aeruginosa</i> Kützinger emend. Elenkin	Loe Pool, Cornwall, UK (Grid ref. SW646245) CCAP 1450/6 Sciento (A535)
	<i>Synechococcus</i> sp.	AK1 Weed species

2.3.2.1 Straw assay

Excess water was blotted off the decomposing straw using paper tissue and cut into pieces ≤ 5 mm in length. The required wet mass of straw was weighed ($\pm 5\%$) and placed into flasks containing the growth medium. Each treatment was replicated five times unless otherwise stated. The dry mass of straw was determined by drying samples in a vacuum desiccator over silica gel, until mass was constant. The mean mass of dry straw was found to be ca. 10% of wet mass.

2.3.2.2 Oak leaf assay

After vigorous stirring, samples of decayed leaves and liquor (water in which they were decomposed) were strained through a 1.5 mm sieve to obtain liquor containing fine particles of the decomposed oak leaves. Previous work had shown the inhibitory activity to be concentrated in the fine particulate organic matter (FPOM) (I. Ridge, pers. comm.). The liquor was diluted to obtain a range of concentrations of FPOM and nutrients were added to make the required growth medium. *Nitzschia filiformis* var. *conferta* was actually isolated from tanks of decaying oak leaves, previously decomposed in the light, therefore some flasks were not inoculated. Final results were then adjusted by subtracting cell counts without inoculation in proportion to the amount of oak leaf material present in each treatment. These adjusted values could then be used for comparison with the control values. The dry mass of FPOM in 50 ml of sieved liquor was determined by filtering through a GF/C glass fibre filter (Whatman) and dried as with the barley straw. 50 ml of sieved liquor contained ca. 30-40 mg FPOM.

2.3.3 Biomass quantification

The correct way to measure biomass is to measure the dry mass of algae left after a period of growth. Due to the small amounts of algae being grown and the presence of plant litter in the cultures, the measurement of dry mass was not possible and biomass had to be

quantified using other techniques. The biomass estimation method and length of assay for each species are shown in Table 2.6.

Biomass estimation method	Species	Growth medium	Duration of assay (days)
Haemocytometer (cell count)	<i>Nitzschia filiformis</i> var. <i>conferta</i>	x3 DM*	4
	<i>Chlorella vulgaris</i>	JM1	4
	<i>Stichococcus bacillaris</i>	JM1	5
	<i>Scenedesmus quadricauda</i>	JM1	†
	<i>Scenedesmus subspicatus</i>	JM1	4
	<i>Microcystis aeruginosa</i>	JM1	4
	(Strains-AK1, CCAP 1450/6, Sciento)		
	<i>Euglena gracilis</i>	EG	6
Sedgewick-Rafter cell (cell count)	<i>Tabellaria flocculosa</i>	WC	5
	<i>Asterionella formosa</i>	DM	4
	<i>Staurastrum pingue</i>	WC	7
	<i>Cosmarium biretrum</i>	WC	7
	<i>Closterium ehrenbergii</i>	WC	8
	<i>Pediastrum boryanum</i>	JM1	4
Chlorophyll <i>a</i>	<i>Spirotaenia erythrocephala</i>	WC	8
	<i>Microcystis aeruginosa</i> (Loe Pool)	JM1	4
	<i>Aphanizomenon flos-aquae</i>	JM1	8
	<i>Anabaena flos-aquae</i>	JM1	8
	<i>Anabaena cylindrica</i>	JM1	8
	<i>Oscillatoria animalis</i>	JM1	8
	<i>Oscillatoria redekei</i>	JM1	8
	<i>Synechococcus</i> sp.	JM1	4

Table 2.6 Biomass quantification method and the time course of a typical assay for each species. * *Nitzschia filiformis* was grown in triple strength medium. † *S. quadricauda* assay was extended (see section 3.3.2)

Footnote: The assay for *Hydrodictyon reticulatum* was carried out differently (see section 3.3.2)

2.3.3.1 Cell counting

Nyholm & Kallqvist (1989) consider cell counting to be one of the most accurate measurements of biomass. The presence of plant litter in these assays precluded the use of electronic cell counters, so manual counting using a light microscope had to be used. In addition to being a good measure of biomass cell counting has other advantages. It allows the experimenter to see if any contaminants are present in the culture and to see if there has been any cytological, morphological, or size change in the species under test. Care must be taken to check that cell volume remains constant in the controls as well as in the toxicant-treated cells. Changes in cell volume may conceal true biomass changes since, for example, large numbers of small cells may have the same biomass as smaller numbers of larger cells. Through manual counting on a light microscope any cell size changes would be evident and could be measured, with the assay results adjusted accordingly. Other problems exist where cells are very small, making manual counting a laborious procedure since the focus has to be constantly adjusted to see small cells which do not settle out quickly. For the purposes of this study, where cultures contained much plant litter debris, manual counting was still considered the most accurate measure of biomass and was used wherever possible.

Method for cell counting

Smaller sized algae were counted using a haemocytometer of the Neubauer ruling type (Fisher) and larger cells were counted using an inverted microscope and a Sedgewick-Rafter counting cell (Graticule). After thorough shaking, one sample was taken from each flask and counted accordingly. Multiple sampling of each flask had shown little variability (results not shown and E. O' Connell, pers. comm.) and was considered unnecessary since five flasks were used for each treatment.

Haemocytometer: Counts were carried out in the four corner squares of the haemocytometer, each of which contained a volume of 10^{-4} ml. The mean number of cells for one square was calculated and the number of cells ml^{-1} calculated accordingly for each flask.

Sedgewick-Rafter cell: The Sedgewick-Rafter cell contained a 1 ml sample. Counts were carried out in five vertical columns of eighteen squares, each evenly spread across the whole of the counting chamber, and the mean calculated.

2.3.3.2 Chlorophyll extraction

Chlorophyll extraction can be used for estimating biomass in filamentous and colonial algae where cell counting is either not possible or the length of time needed to carry out counts makes the method unworkable. Chlorophyll *a* is present in all autotrophic algae and cyanobacteria and can be measured spectrophotometrically after extraction. It is an appropriate method for the rapid estimation of algal biomass, as long as the cultures remain unialgal: contamination of cultures with other species would give spurious results for the actual alga being tested. For this reason, oak leaves and barley straw were incubated in the dark to prevent the growth of resistant species which could affect the results.

Chlorophyll *a* content can be altered by a number of factors such as irradiance, day length, temperature and nutrient status (Foy, 1987), but these were of no consequence in this study since they were kept constant at all times. Shading by the plant litter may have lowered irradiance to the algae, but this was thought to be unlikely since Gibson *et al.* (1990) showed no shading effects with up to 4000 g dry mass straw m⁻³ medium. Care has to be taken to check that the action of plant litter does not affect chlorophyll *a* concentrations in the cell, though for *Chlorella vulgaris* and *Microcystis aeruginosa* chlorophyll *a* has been shown to correlate directly with cell counts (Pillinger, 1993; J. Walters, pers. comm.).

Various solvents have been used to extract chlorophyll *a* though Marker *et al.* (1980) recommended methanol over all other solvents and it has been used in previous studies of this type (Gibson *et al.*, 1990; Pillinger, 1993). Absolute values of chlorophyll *a*

were not so important to the study since comparisons were made only against a control, hence the type of solvent used was not critically important to the bioassay results.

Method for chlorophyll extraction

The method used for chlorophyll extraction was based largely on the method of Pillinger (1993) and Pillinger *et al.* (1994, 1995). At the end of an experiment the contents of the flask were filtered onto 70 mm GF/C glass-fibre filters (Whatman) by means of a vacuum pump and Buchner funnel. The filter and residue were transferred to a universal bottle (Merck) and methanol added (10-20 ml). The lid was screwed on and the bottle placed in a water bath at 80°C for 2-3 minutes to boil the methanol. Cooling of the solution was carried out in the dark to prevent degradation of the chlorophyll. If the solution was not optically clear it was filtered through GF/C again and an aliquot of the solution was transferred to a disposable cuvette (10 mm light path, Merck). Absorbance was measured on a SP6-550 UV/VIS spectrophotometer (Pye Unicam) at 665 nm and corrected by subtracting a reading at 750 nm to account for background turbidity. Readings at the same wavelengths were also taken after the methanol extract had been acidified with 1 M HCl, giving a final concentration of 3 mM HCl, to correct the sample for the degradation products of chlorophyll, specifically phaeophytin *a* (Marker *et al.*, 1980). The amount of methanol was adjusted according to the species used and the duration of the assay to give absorbance values in the range 0.1-0.8, which was the range over which absorbance was directly related to pigment concentration (Marker *et al.*, 1980; J. M. Pillinger, pers. comm.). Absolute values of chlorophyll *a* were calculated following the equation of Jones (1979):

$$\text{Chl } a \text{ (}\mu\text{g l}^{-1}\text{)} = 12.98 (A - A_a) \times \frac{\text{volume extract (ml)}}{\text{volume sample (l)}}$$

Where A is the corrected absorbance at 665 nm before acidification

Where A_a is the corrected absorbance at 665 nm after acidification

2.4 Expression of results

Throughout this thesis the estimated biomass (or yield) of algae at the end of an assay was used for comparison between the control (in the absence of plant litter) and treated cultures. All results are expressed as 'growth as a percentage of the control' following the formula:

$$\frac{\text{estimated algal biomass of treated culture}}{\text{estimated algal biomass of control culture}} \times 100$$

This allowed inter and intra-species comparisons to be made without directly comparing cell counts or absolute masses of chlorophyll *a*. Experiments were replicated at least twice and comparisons were made by means of student's t-test or an analysis of variance where different masses of plant litter were used in an assay.

Chapter 3

Bioassay Results

Kingdom **Plantae**

3.1 Division Heterokontophyta

Class **Bacillariophyceae (diatoms)**

The cell wall consists predominantly of polymerized silicic acid but also contains the proteins, lipids and polysaccharides present in the organic wall of other algal groups (Hoek *et al.*, 1995). The silicon wall or frustule consists of two halves, the hypotheca and the epitheca, which are joined together by girdle bands (epicingulum and hypocingulum). The two major groups of the Bacillariophyceae are the pennate diatoms and the centric diatoms. In many pennate diatoms the frustule has a longitudinal slit called the raphe which usually lies along the apical plane. The raphe is involved in locomotion so diatoms without raphes cannot move actively; centric diatoms are, therefore, largely non-motile.

Reproduction

Prior to division the cell contents swell forcing the epitheca and hypotheca apart. Mitosis then takes place, followed by division of the protoplast and subsequently the formation of new wall elements. The newly synthesized half of the cell in each daughter cell is always a hypotheca (smallest half), whereas the parental theca always forms the epitheca regardless of whether it was originally an epi or hypotheca. The consequence of this type of cell division is that one daughter cell is always smaller than the parent. This means that the average cell size of a diatom population decreases with each successive cell division, although some species have a fairly elastic girdle allowing them to maintain their size (Boney, 1989; Hoek *et al.*, 1995). With continuing cell division the cells eventually reach a minimal size and may then form auxospores. Auxospores are usually formed via sexual

reproduction (the auxospore being the zygote) and they do not possess siliceous walls. Expansion of the auxospore reverses cell size reduction and produces larger cells.

Diatom biomass is more significant in the spring in the U.K. since certain species can grow to nuisance proportions in the early part of the year, before becoming limited by nutrients, usually silicon (see section 1.2.2.1). As mentioned previously (section 1.5) it had been suggested that diatoms appear to be resistant to the inhibitor(s) from plant litter since one species was found to grow in tanks of rotting barley straw (reported in Ridge *et al.*, 1995). However, in field trials in a reservoir, Barrett *et al.* (1996) found that the planktonic diatoms *Asterionella formosa* and *Tabellaria* sp. were suppressed after the introduction of barley straw, although no true comparable control was available in this study and cell counts had to be compared to those obtained from the reservoir in previous years. The presence of a unique type of wall (silicon frustule) and the fact that one species appeared to be resistant to inhibitor(s) suggested that the cell wall may play some part in resistance to litter-derived inhibitors. Initial investigations concentrated on the diatoms because of their cell wall structure and to see if laboratory investigations resembled results obtained in the field.

3.1.1 Order Pennales

Asterionella formosa

This species is a planktonic, colonial alga forming 'star-shaped' colonies (Plate 3.1) with the ends of the cells attached to each other by mucilage pads. It is common in most water bodies but is particularly abundant in meso-to eutrophic ponds and lakes in the spring and autumn (see section 1.2.2.1) (Pentecost, 1984). It is frequently associated with filter clogging and has occasionally been reported as causing taste and odours in water (Round, 1984; Palmer, 1980).

Tabellaria flocculosa

In this planktonic, colonial species (Plate 3.1), the ends of the cells are attached to each other by means of mucilage pads. It grows in oligotrophic to eutrophic waters where it is almost ubiquitous in Britain (Bellinger, 1992), but tends to favour lower pH than

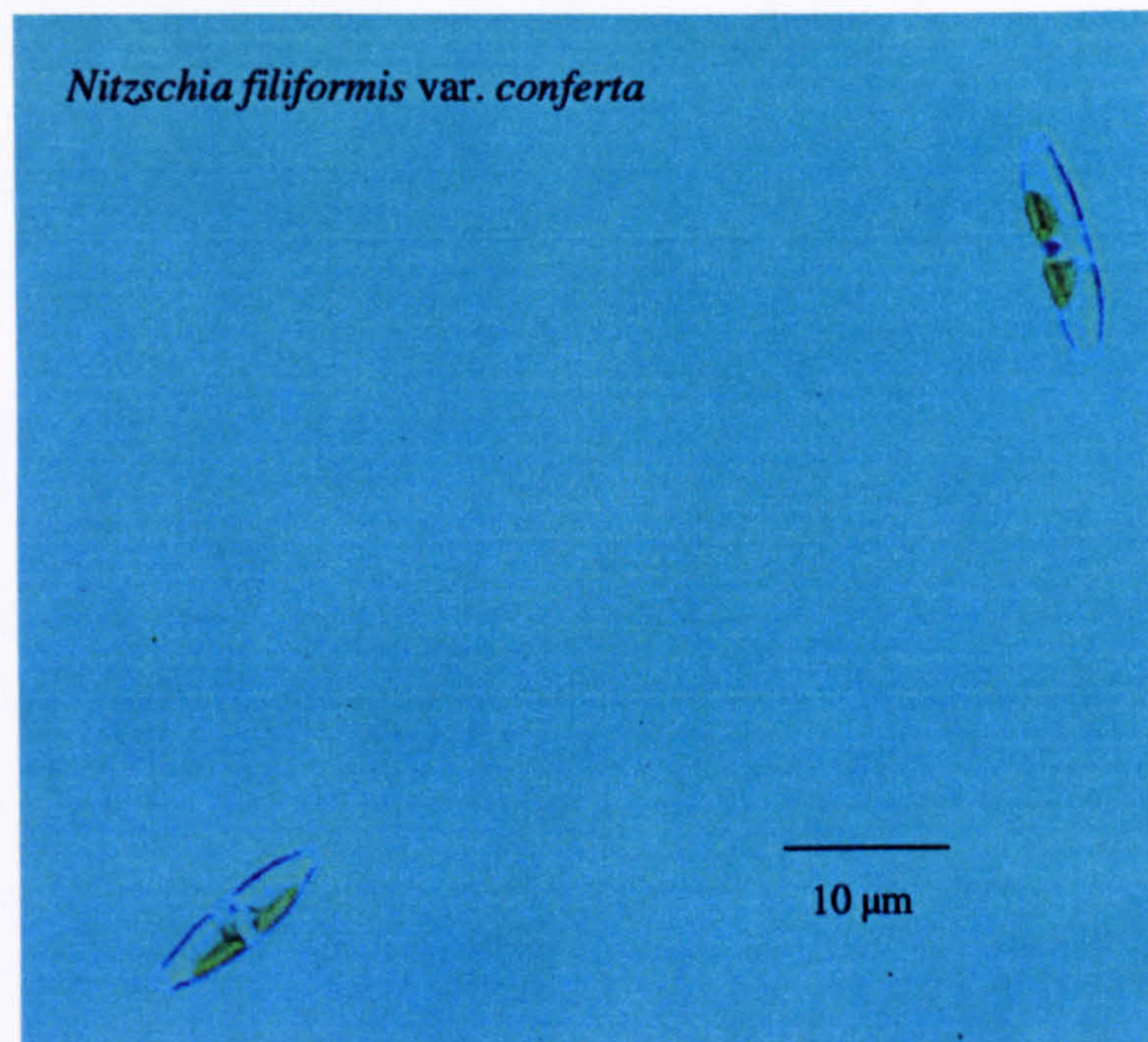
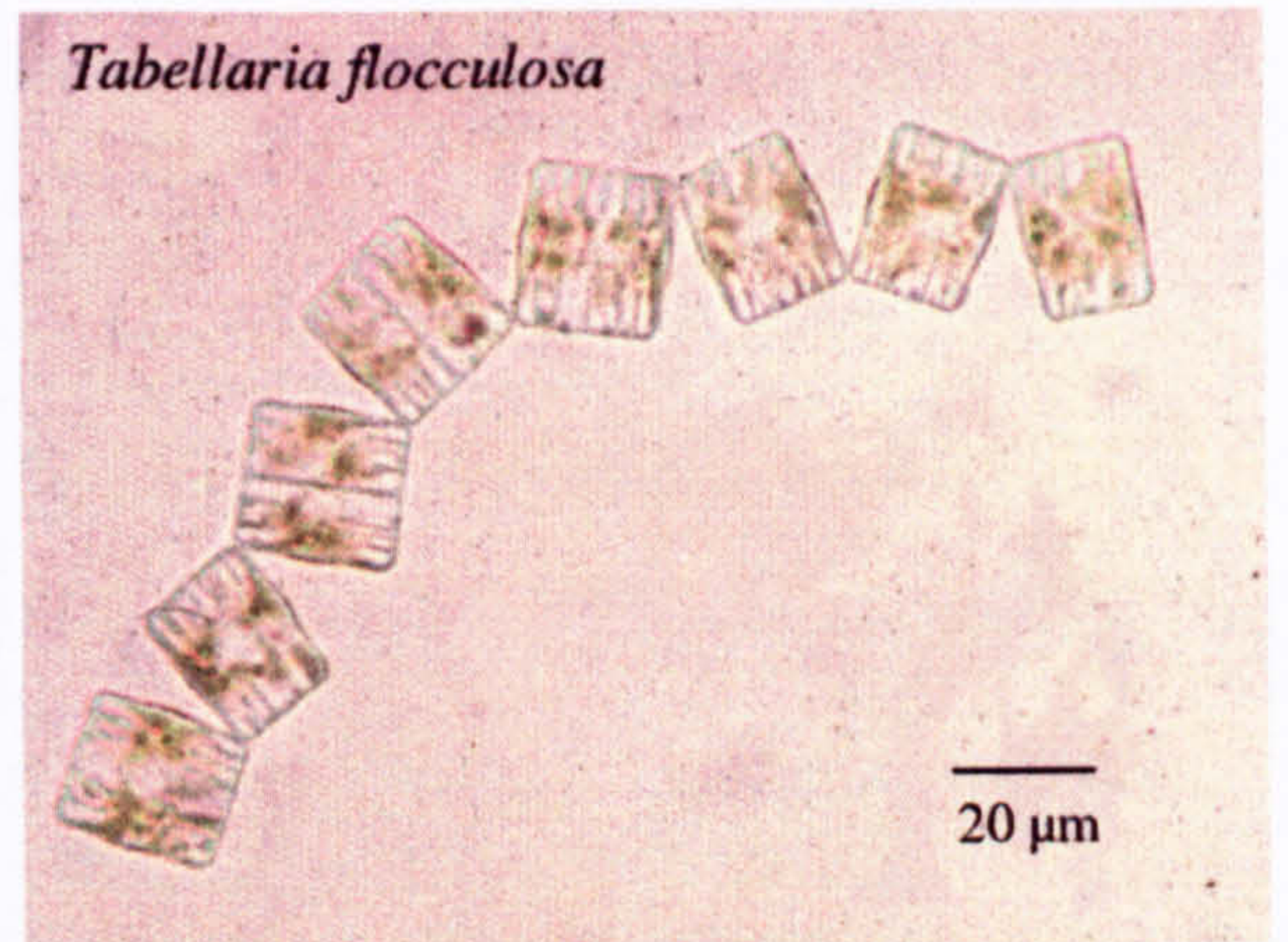
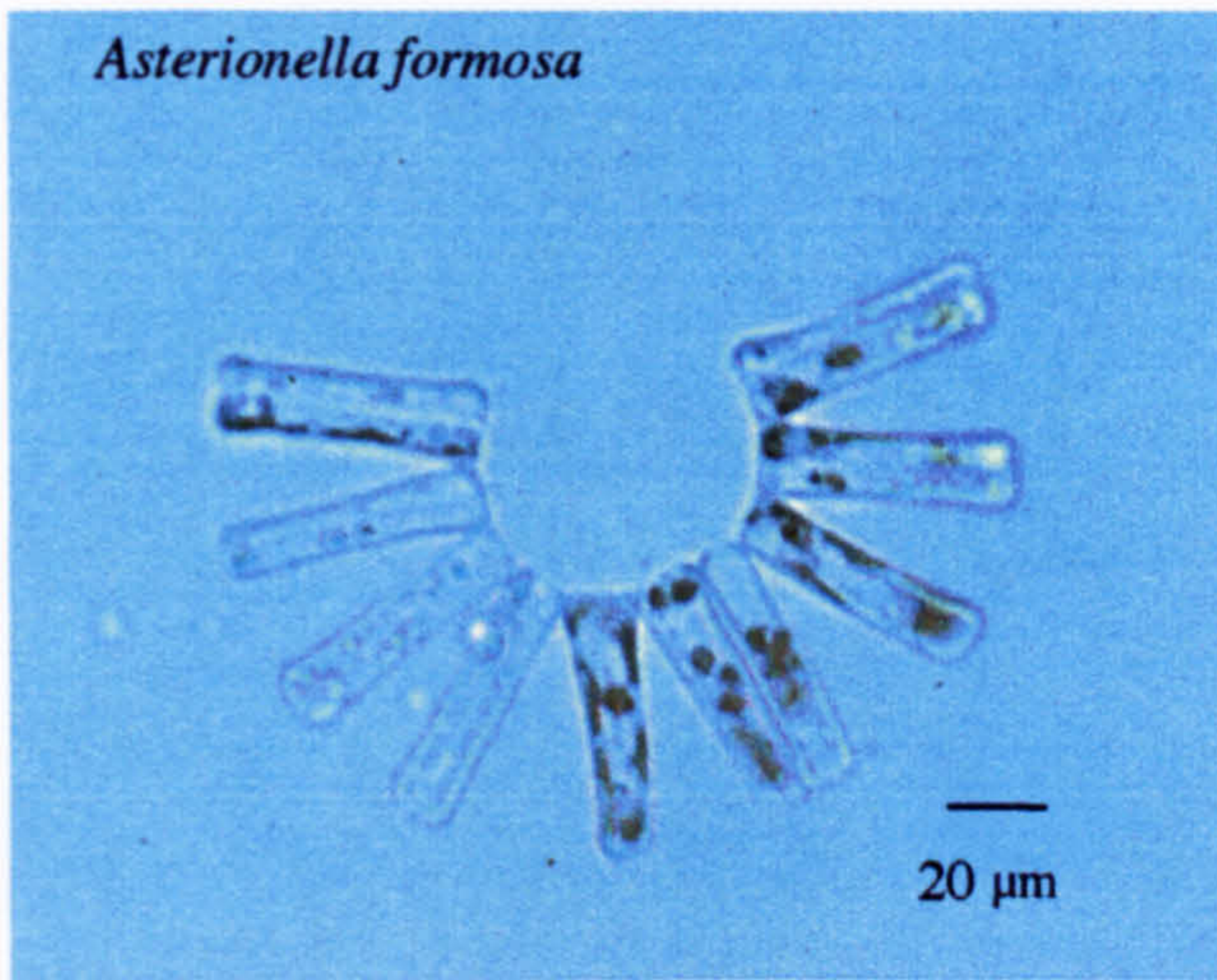


Plate 3.1 Members of the Bacillariophyceae which were assayed.

Asterionella formosa. It can form blooms in spring and autumn which can interfere with the efficient operation of sand filters. The gelatinous attachments of the cells make the chain of cells flexible and help to prevent breakage. These chains may, therefore, be more effective than long filamentous algae in forming slime covers and clogging filters (Palmer, 1980).

Nitzschia filiformis* var. *conferta

The genus *Nitzschia* is very common in highly polluted areas and sewage stabilization ponds (Palmer, 1969, 1980). *N. filiformis* var. *conferta* (Plate 3.1) is a motile, surface-associated species, being commonly found in eutrophic and/or very polluted waters of high conductivity (Kramer & Lange-Bertalot, 1988). This strain was isolated from a tank of decomposed oak leaves which had been left open to the air and to daylight in a greenhouse.

3.1.2 Results

Both barley straw and FPOM derived from decaying oak leaves inhibited the two planktonic diatoms *Asterionella formosa* and *Tabellaria flocculosa* with the latter appearing to be the more sensitive (Figure 3.1), although both species were more sensitive to oak leaf FPOM than barley straw. The 50% inhibition value for *T. flocculosa* is about half that of *A. formosa* with barley straw and about one fifth that of *A. formosa* with oak leaves (Table 3.2 page 86). *Nitzschia filiformis* was not inhibited by either oak leaf or barley straw and growth was stimulated in both cases, although oak leaf FPOM produced the strongest growth promotion.

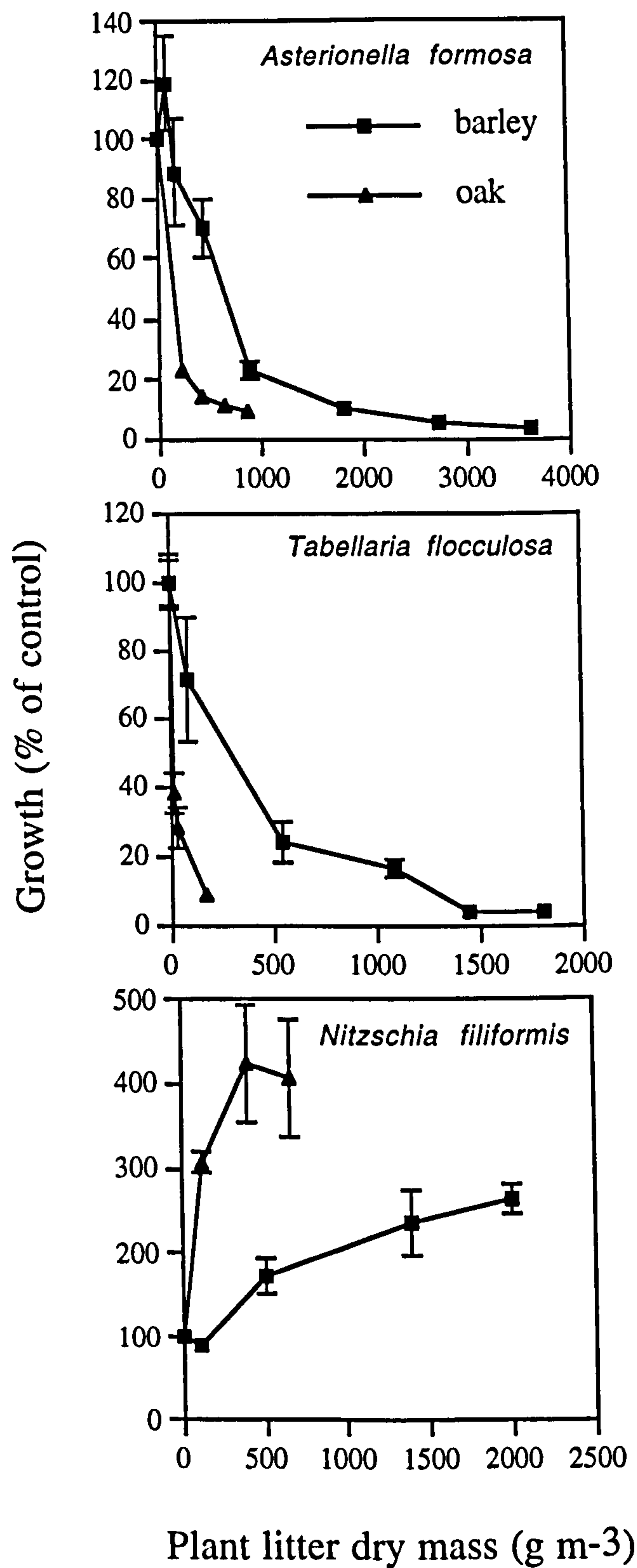


Figure 3.1 Growth of *Asterionella formosa*, *Tabellaria flocculosa* and *Nitzschia filiformis* var. *conferta* with different masses of barley straw and oak leaf FPOM. Values shown are means ($n=5$) \pm SE. All graphs show a significant difference from control values ($p < 0.001$).

3.1.3 Discussion

Decomposing barley straw and oak leaves can inhibit the growth of certain diatoms under the specified laboratory conditions. This is contrary to the observation reported in Ridge *et al.* (1995) which suggested that diatoms as a group may not be inhibited. The current laboratory investigations support the field observations of Barrett *et al.* (1996), who showed that *Asterionella formosa* and *Tabellaria* sp. were inhibited when barley straw was introduced into a reservoir. *N. filiformis* was not inhibited at all, as was perhaps expected in the case of oak leaf, since it was originally isolated from a tank of decomposing oak leaves. It is possible that *N. filiformis* is not inherently resistant to litter-derived inhibitors but the strain used in this investigation had been selected for resistance because of its origin. It would be prudent to test a different strain of the same species, which had not been isolated from tanks of decomposing litter, to see if resistance is an adaptive feature (although from the results presented in this chapter (see Table 3.2 Page 86) other species which have not been isolated from decomposed litter are resistant). Even if not inherently resistant, the use of this strain of *N. filiformis* at least shows that a species of diatom can become adaptively resistant to inhibitors derived from plant litter.

The complete resistance of *N. filiformis* shows that diatoms vary in their susceptibility to litter-derived inhibitors. In this case the major difference between the diatoms appears to be their ecology. *N. filiformis* is a surface-associated diatom whereas the other two diatoms tested, *Asterionella formosa* and *Tabellaria flocculosa*, are planktonic. Whether surface dwelling makes a diatom species intrinsically resistant to decaying plant litter is not known and further tests on a wider range of diatom species are required.

3.2 Division Chlorophyta

Class Zygnematophyceae

Species were chosen from this class because of the differences which occur in the cell walls between those species belonging to the Desmidiales (placoderm desmids) and those species in the family Mesotaeniaceae belonging to the order Zygnematales (saccoderm desmids). Also, small numbers of desmids had been found in ponds which had been treated with barley straw and oak leaves where the growth of *Cladophora glomerata* was shown to be inhibited (I. Ridge, pers. comm.).

The cell walls fall into two main categories depending on the form of the outer layer: those which are electron dense (placoderm desmids) and those which are not (saccoderm desmids) (Brook, 1981). All of the species have a cell wall with an inner-cellulose layer, a mid-layer of cellulose and pectin and an outer-mucilaginous layer or an outer-amorphous layer made up of pectic substances mixed with hemicelluloses (Gerrath, 1993). Although the primary and secondary cell walls of all desmids are fundamentally of the same construction, the distribution of pores and the form and composition of the outer layer conforms to three basic types (Brook, 1981), as shown in Figure 3.2. It was thought prudent to test species which had different types of cell wall and ascertain whether some general feature of cell wall morphology correlated with insensitivity/sensitivity towards decomposing plant litter.

Reproduction

Reproduction is most commonly via asexual vegetative cell division and less commonly by the formation of zygotes and sexual reproduction. Placoderm desmids usually have a constriction in the middle of the cell known as the sinus (for example see *Staurostrum pingue* and *Cosmarium biretrum* on Plate 3.2). The narrow section which joins the two cell halves or semicells is called the isthmus. Cell division occurs by elongation of the isthmus to form a new semicell; a new sinus forms in the plane of the original and the two new semicells separate at the apical walls. In species where the cell is not divided into two semicells the cell contents are similarly divided (see *Closterium ehrenbergii* on Plate 3.2).

Cell division occurs by transverse cleavage of the chloroplast, nuclear division and the subsequent formation of cross walls to produce a new cell. Sexual reproduction in the natural environment is a rarely recorded event (Gerrath, 1993) and was never observed in the cultures used in the present investigations, therefore, it was discounted as a possible action site for the litter-derived inhibitor(s).

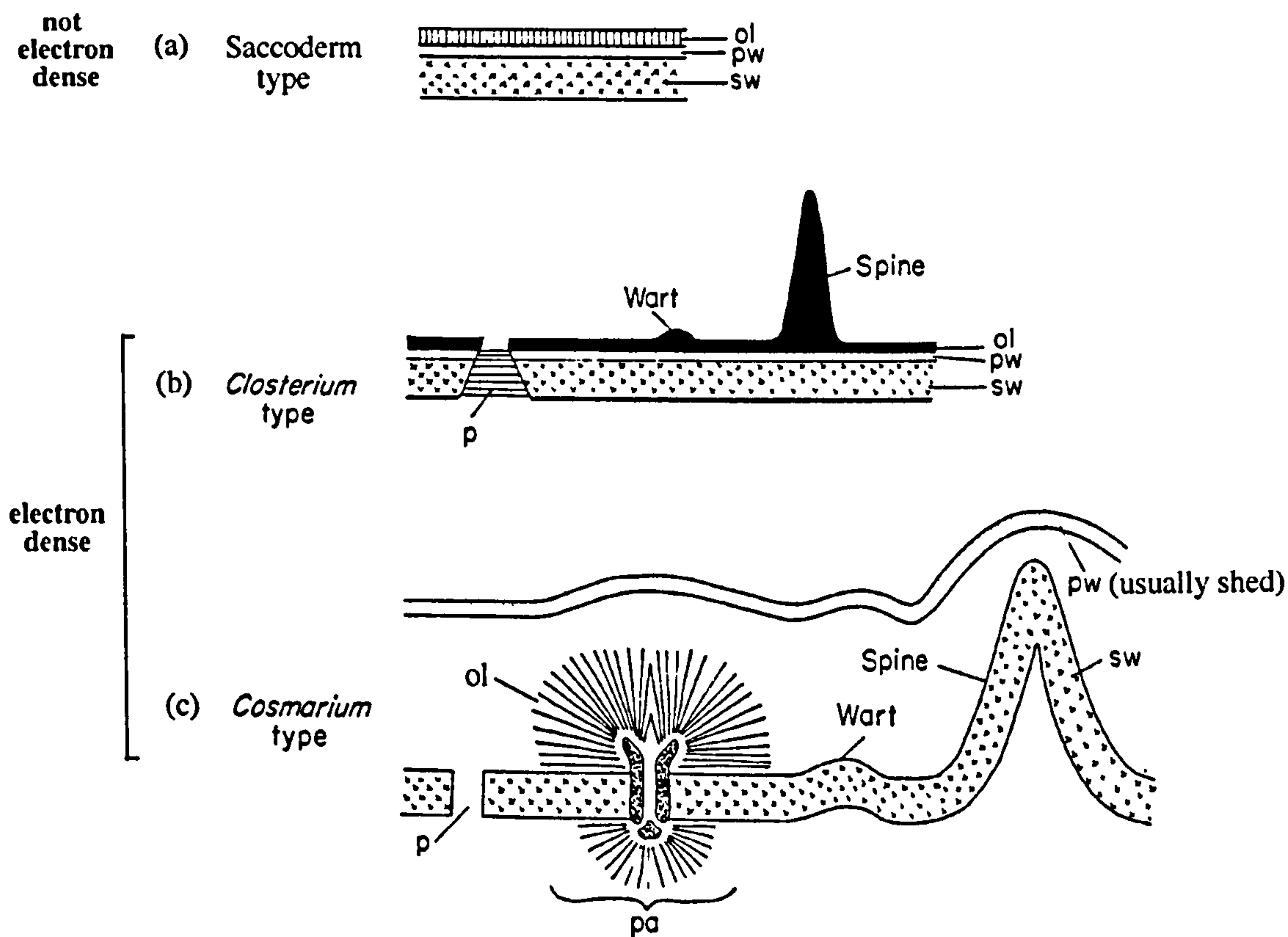


Figure 3.2 Schematic diagram of the three basic types of desmid cell wall. (a) saccoderm-type with no pores (b) *Closterium*-type where pores are open in the outer layer but crossed by fibrils of the primary and secondary wall (c) *Cosmarium*-type has no fibrils extending through the pores but extrudes fibrils which radiate outwards from the pore to form the mucilaginous outer layer. Simpler pores may also be present, occurring in a regular pattern amongst the large more complex ones. The primary wall is usually shed after development of a thick secondary wall (modified from Brook (1981)). ol = outer mucilaginous layer; pw = primary wall; sw = secondary wall; p = pore; pa = pore apparatus.

Desmids are generally more prevalent in acidic waters (Brook, 1981; Gerrath, 1993, Hoek *et al.*, 1995) so it was necessary to test species which were able to grow in the basic conditions under which the bioassays were carried out.

3.2.1 Order Desmidiales (Placoderm desmids)

Family Closteriaceae

Closterium ehrenbergii

This is a single-celled species which is epiphytic (Plate 3.2) and often occurs in pools, ditches and slow-running streams where it can sometimes grow in abundance (West & West, 1904). The presence of *C. ehrenbergii* in an algal community can indicate increased levels of nutrients (Gerrath, 1993). It grows naturally in mildly acidic to mildly basic waters (Lenzenweger, 1996) and can be easily grown in culture under basic conditions (Nawata, 1988), thus making it an ideal desmid species to test under the conditions employed in the plant litter assays.

C. ehrenbergii has the *Closterium*-type of cell wall whereby open pores are limited to the outer-amorphous layer of the cell (see Figure 3.2) and are crossed by fibrils in the primary and secondary wall layers (Brook, 1981; Gerrath, 1993). The pores are thought to enable the cell to secrete mucilage and connect with the extracellular environment (Brook, 1981).

Family Desmidiaceae

Cosmarium biretrum

In the U.K. *C. biretrum* (Plate 3.2) is found principally in the marshes and ditches of low-lying areas. It is often found in abundance among various *Potamogeton* species in land drains of the east of England (West & West, 1912) and is commonly found in eutrophic waters (Coesel & Wardenaar, 1990).

Although *C. biretrum* has the electron-dense type of wall characteristic of placoderm desmids, there are some differences between it and *Closterium ehrenbergii*. *C. biretrum* has the *Cosmarium*-type of cell wall whereby the primary wall is present only in newly expanded semicells and is discarded after secondary wall formation is completed

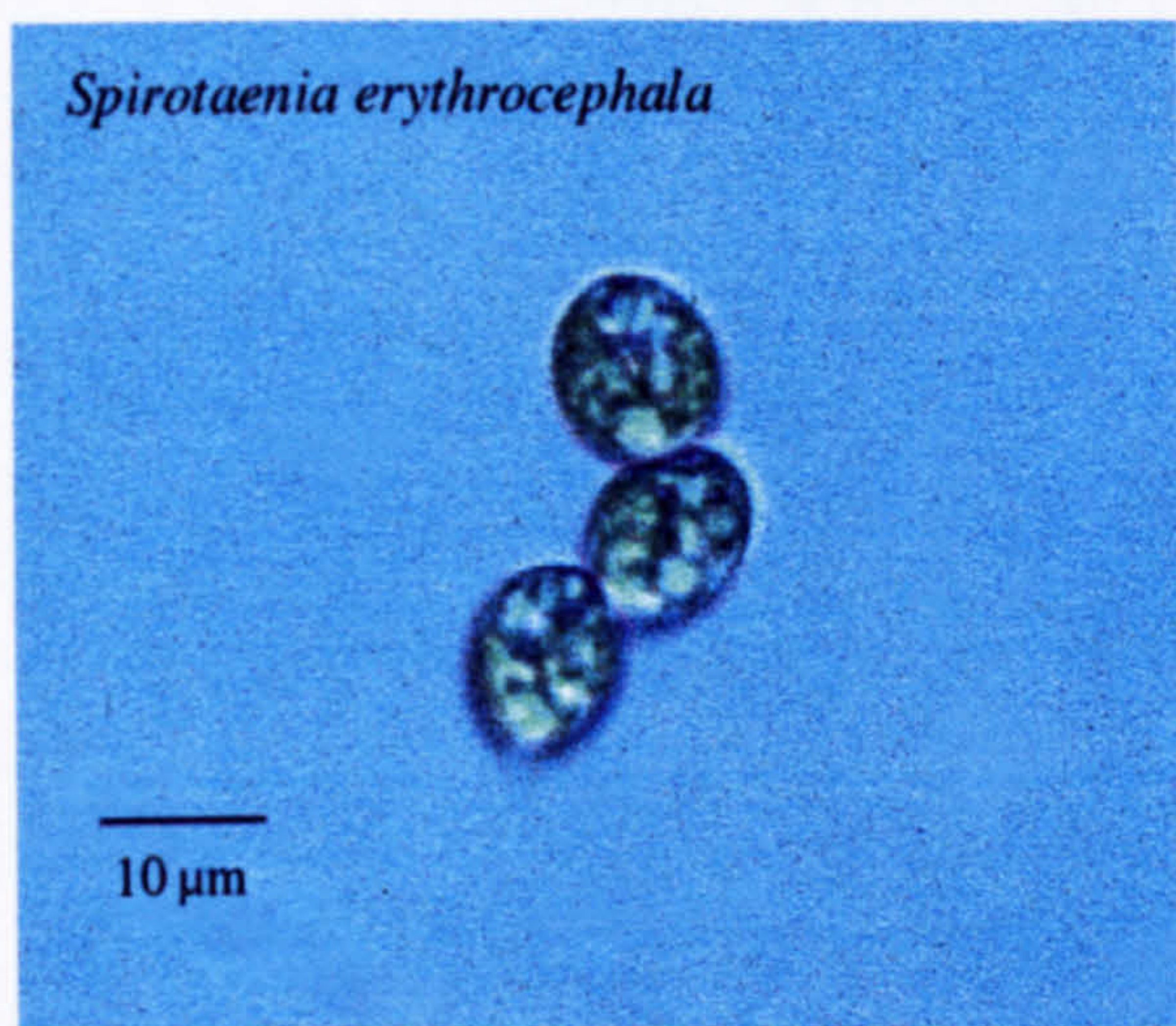
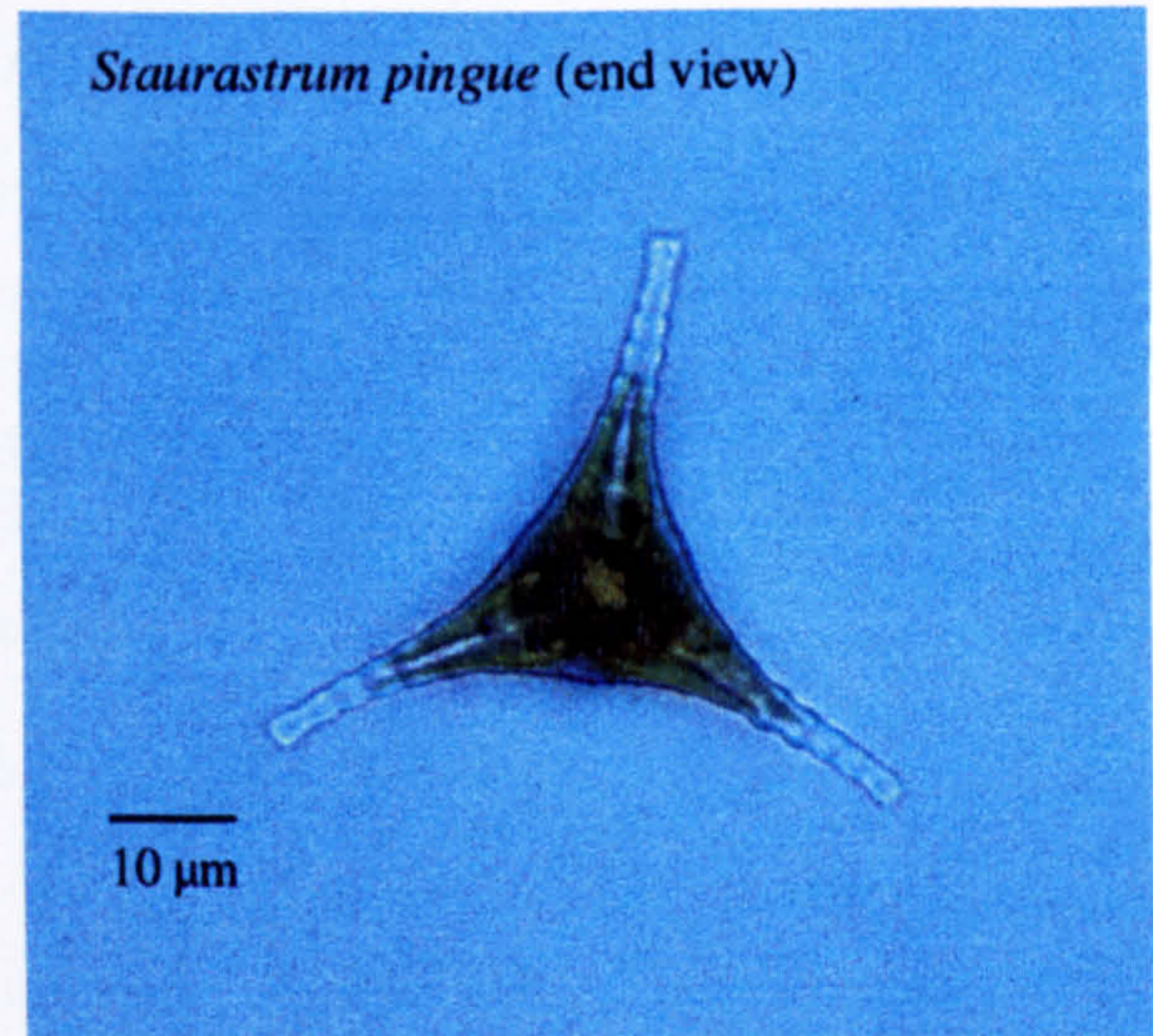
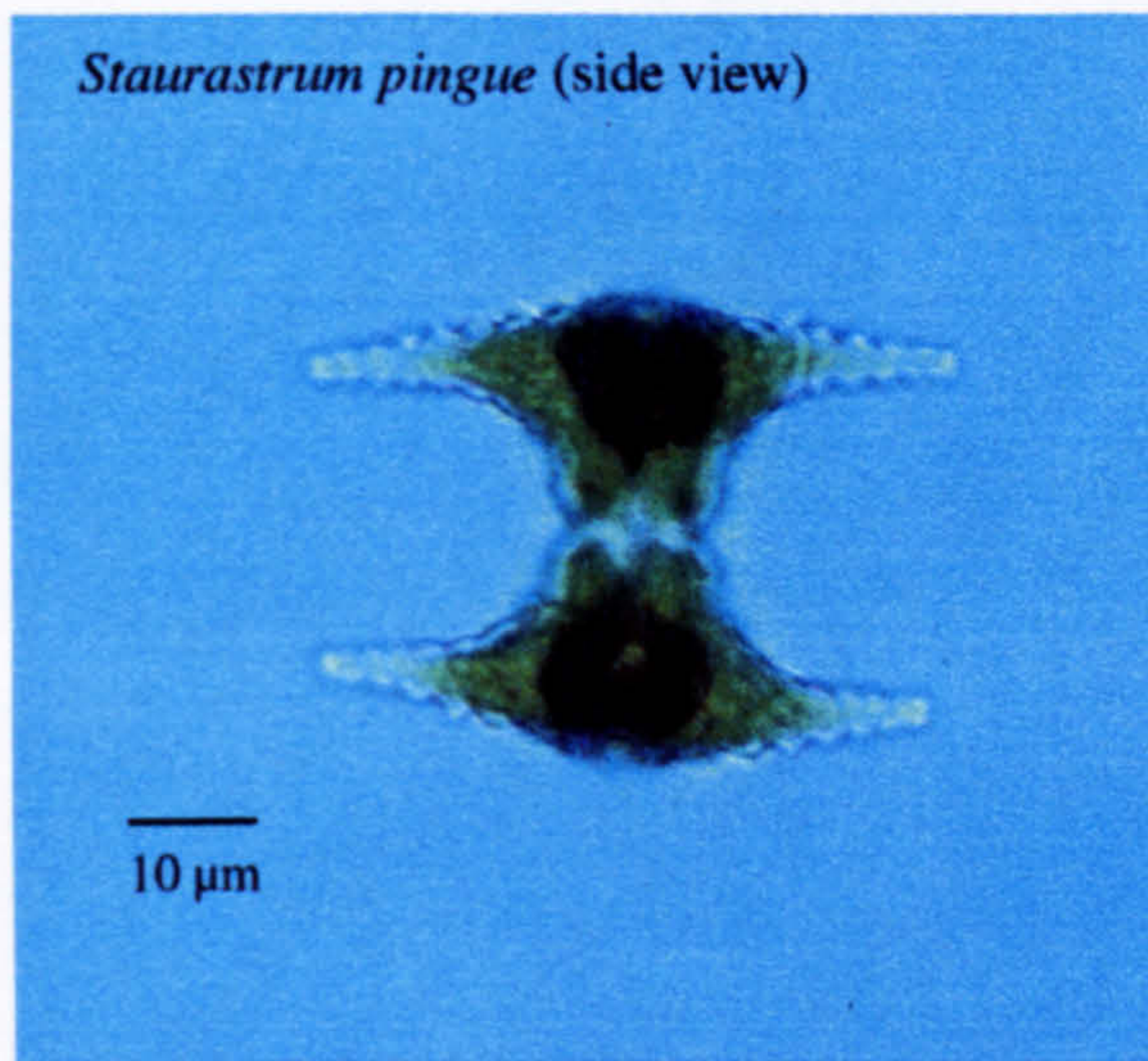
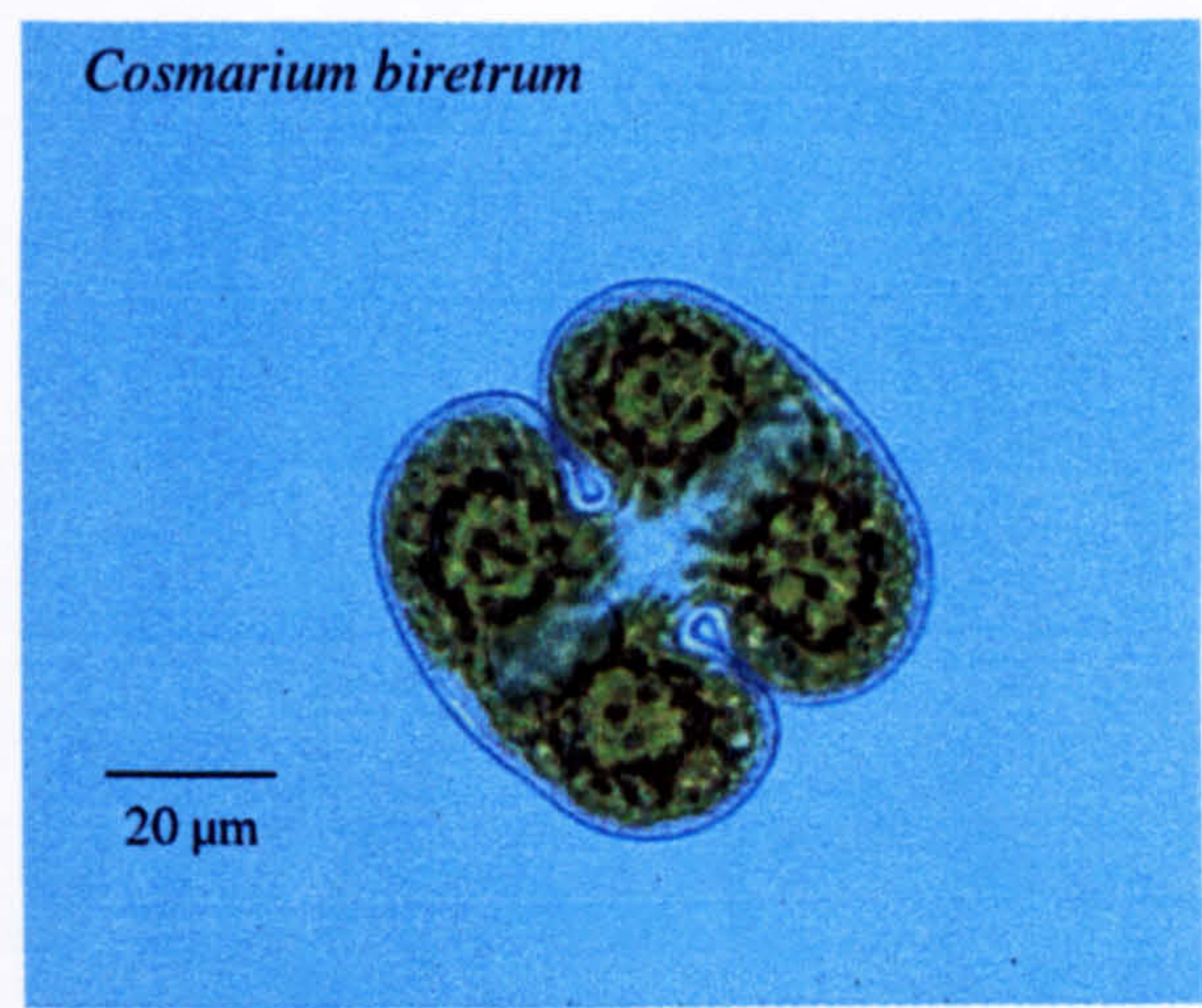
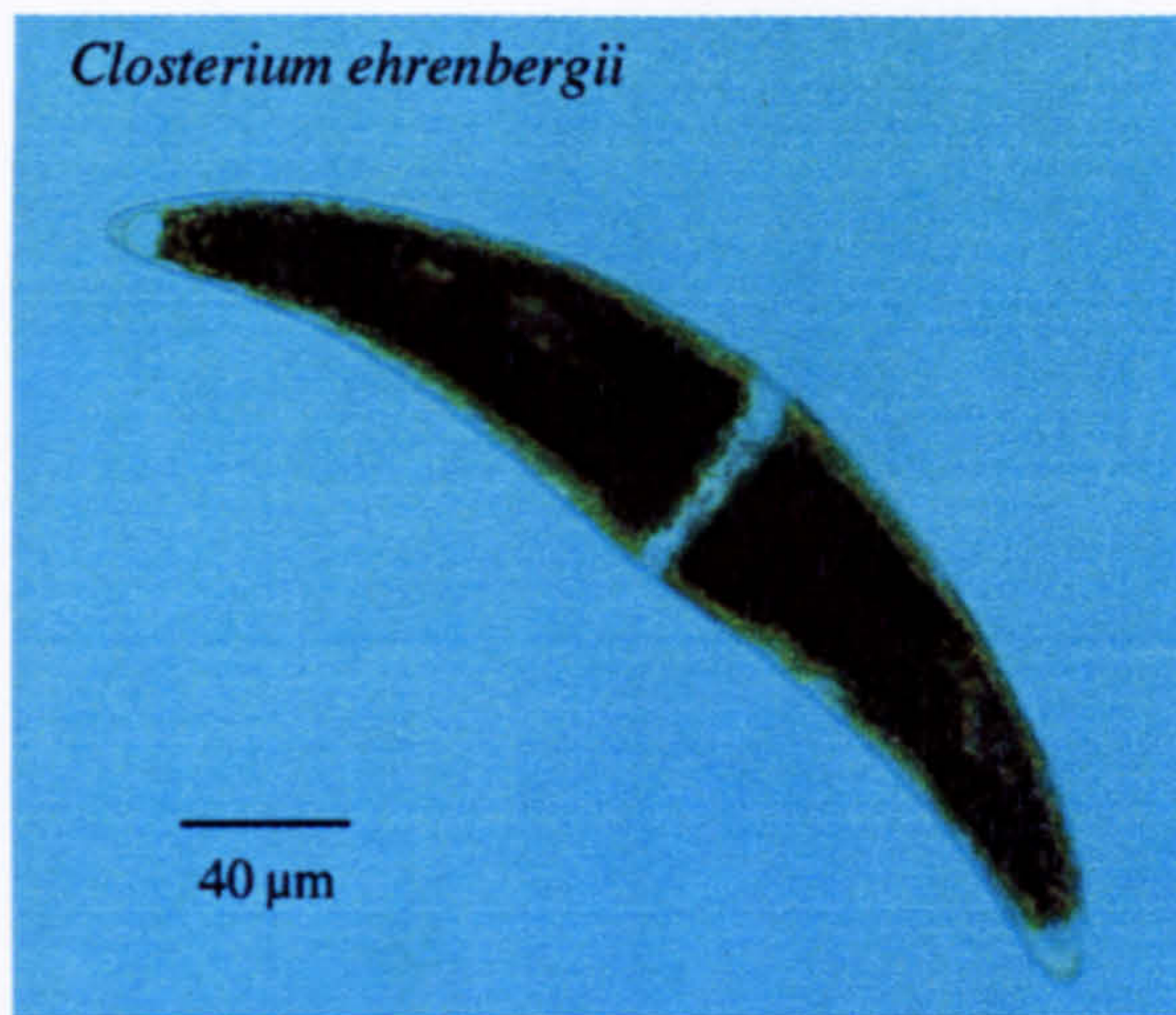


Plate 3.2 Members of the Zygnematophyceae which were assayed.

(see Figure 3.2(c)); the secondary wall is then covered with an outer mucilage sheath (Brook, 1981). Unlike the *Closterium*-type of wall the pores in the secondary wall of *Cosmarium*-type desmids are not crossed by fibrils, but they are still thought to act as openings to the extracellular environment through which external sheath segments can be formed (Brook, 1981; Gerrath, 1993)

Staurostrum pingue

This is a single-celled, planktonic alga, having a sculptured cell wall of the *Cosmarium*-type (see Figure 3.2). It possesses projections or processes which arise from each semicell (Plate 3.2). *S. pingue* was thought ideal to test with plant litter since it is one of the few desmid species to form blooms in very eutrophic lakes (Brook, 1981).

3.2.2 Order Zygnematales

Family Mesotaeniaceae (Saccoderm desmids)

Spirotaenia erythrocephala

Unlike the placoderm desmids, the outer-layer of *Spirotaenia erythrocephala* is permeable to electrons and has no pores extending through the cell wall (see Figure 3.2(a)). *S. erythrocephala* is a distinctly oligotrophic species preferring acidic conditions and as such is often found amongst the tychoplankton of *Sphagnum* bogs (A. J. Brook, pers. comm.). This species was chosen because it was a readily available representative of the saccoderm desmids. Although preferring acidic conditions in the field, it appeared to grow well in laboratory culture at a pH of 7.2, allowing comparisons to be made with the other desmids being tested.

3.2.3 Results

Barley straw inhibited all of the desmids tested with *S. pingue* requiring the most straw to achieve 50% inhibition of growth (Figures 3.3-3.6), although all the desmids are classed as very susceptible (see Table 3.2 page 86). Oak leaf FPOM inhibited both the desmids

tested, with more oak leaf FPOM being required for 50% inhibition of *S. pingue* than for *C. biretrum*, thus mirroring the results obtained with barley straw.

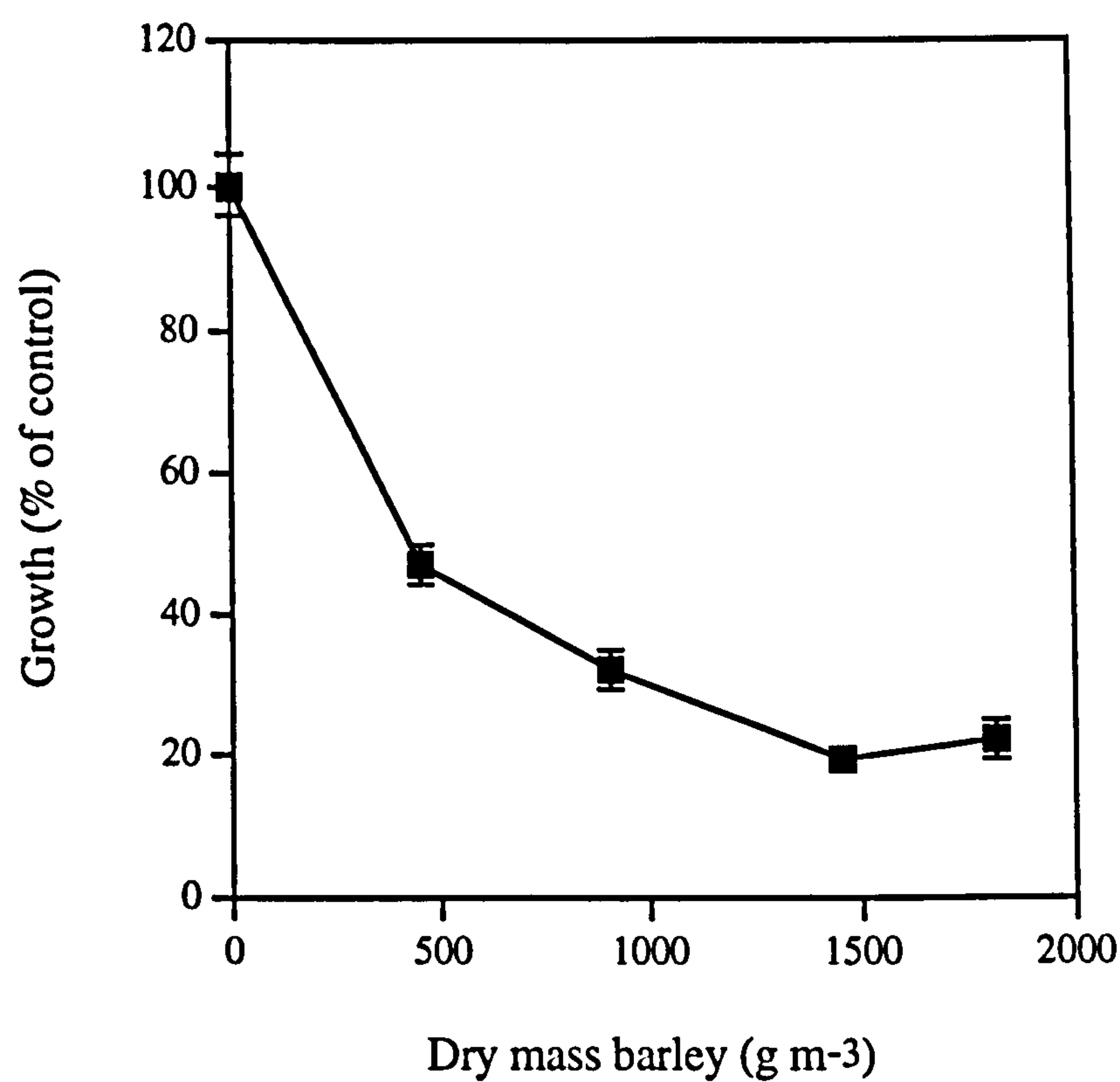


Figure 3.3 Growth of *Closterium ehrenbergii* with different masses of barley straw. Values shown are means (n=5) \pm SE. Inhibition is significant $p < 0.001$.

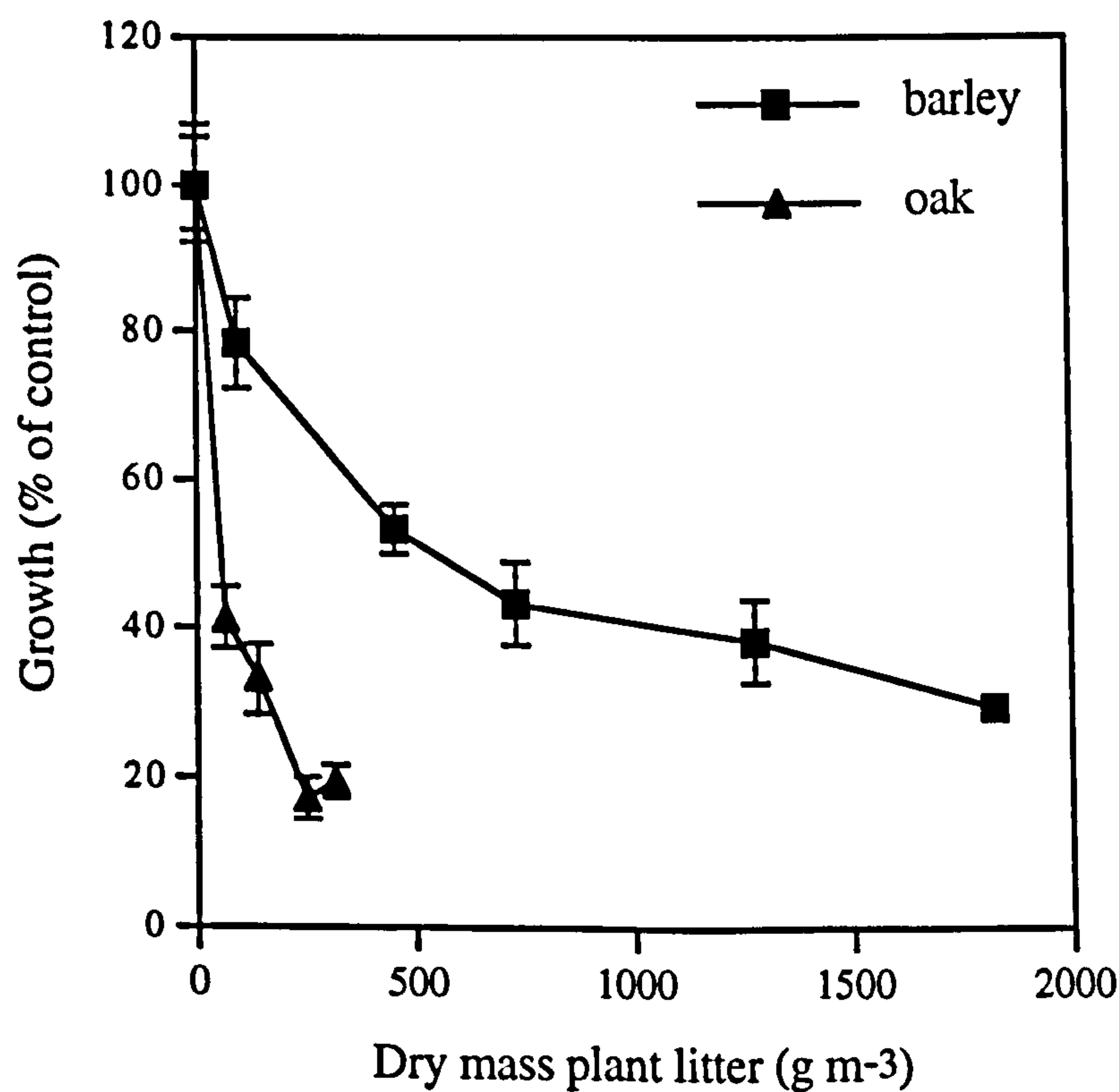


Figure 3.4 Growth of *Cosmarium biretrum* with different masses of oak leaf FPOM and barley straw. Values shown are means (n=5) \pm SE. Inhibition is significant $p < 0.001$.

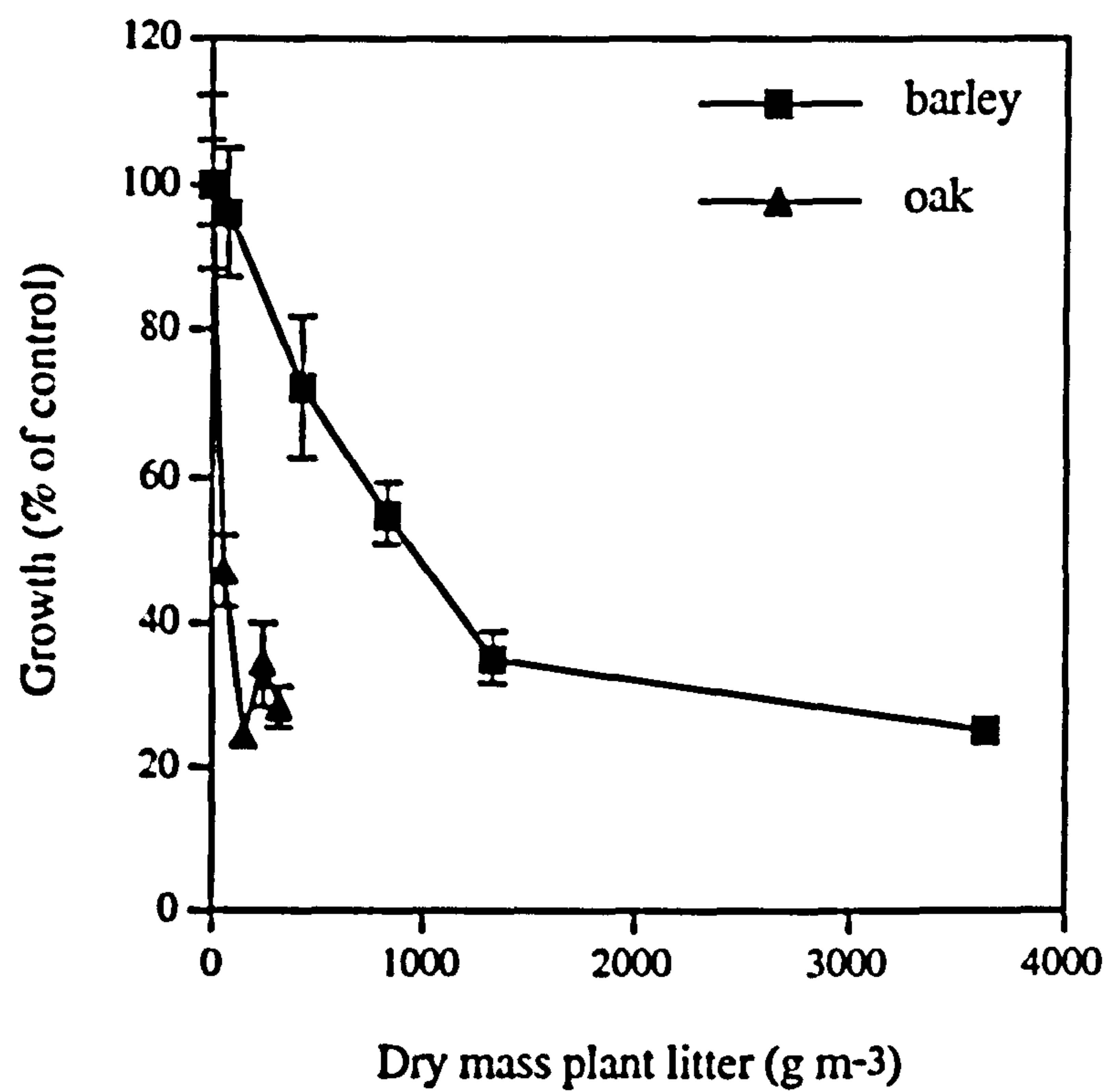


Figure 3.5 Growth of *Staurastrum pingue* with different masses of oak leaf FPOM and barley straw. Values shown are means (n=5) \pm SE. Inhibition is significant $p < 0.001$.

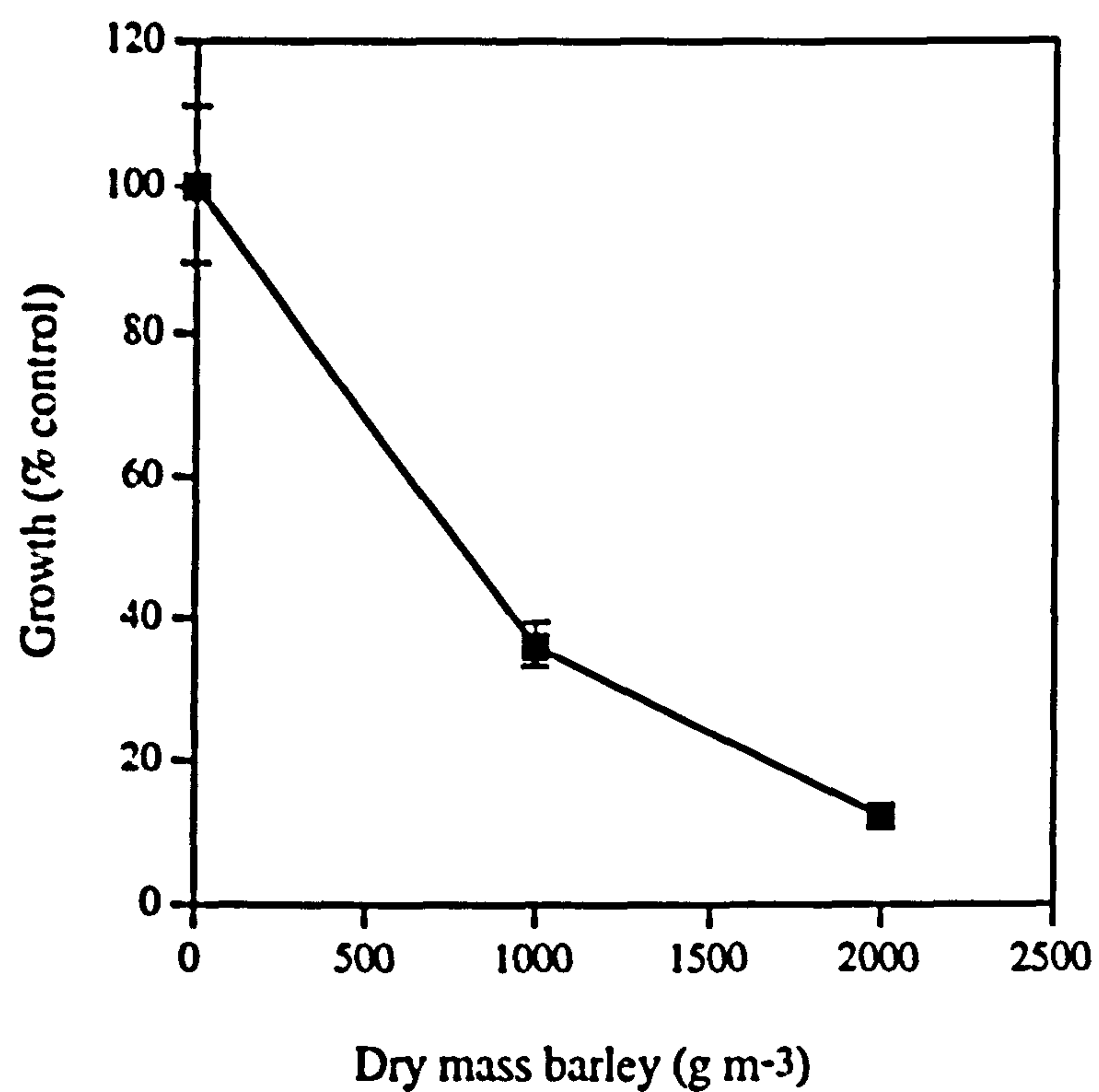


Figure 3.6 Growth of *Spirotaenia erythrocephala* with different masses of barley straw. Values shown are means (n=5) \pm SE. Inhibition is significant $p < 0.001$.

3.2.4 Discussion

Staurostrum pingue requires the highest amount of plant litter of all the desmids to achieve a 50% inhibition in growth. The amount of plant litter required may have been lower if actual biomass measurements had been possible. From Plate 4.1 (page 91A) it can clearly be seen that the process length of *S. pingue* is dramatically reduced in the presence of decomposed plant litter (see section 4.2.1 for further details). Counting the number of treated cells will not give a true comparison with the controls, since the treated cells have shorter processes and therefore a lower biomass than untreated cells. This reduction in process length will be reflected in the final results by showing that a higher mass of plant litter is required to achieve 50% inhibition of growth. Comparisons between controls and treated cells should ideally compare actual biomass rather than estimates based upon cell counting; this was not possible for reasons explained previously (section 2.3.3).

Cell wall type

Although there are differences in the amount of plant litter required to achieve a 50% inhibition of growth (see Table 3.2 page 86), the differences are not particularly large (all the desmids are ranked as very susceptible to barley straw), even if the morphology of *S. pingue* cells is not taken into account. The different cell wall and pore types of the placoderm and saccoderm desmids appear not to be significant in determining the sensitivity of the desmids, although only one species was tested of the saccoderm type (*Spirotaenia erythrocephala*). There may possibly be subtle differences in sensitivity related to the different cell wall types, but this could not be determined to an acceptable degree of accuracy because of the variable nature of the plant litter (see section 1.3.1.1).

Mucilage

Functions ascribed to the mucilaginous layer of the desmids include the trapping of nutrients, its action as a buoyancy aid to slow down rates of sinking in planktonic desmids,

and its use as a protective material, especially in desmids of sub-terrestrial habitats (Boney, 1980, 1981; Brook, 1981).

Mucilage production in all algae is often diminished in the laboratory, compared to field conditions, since there are fewer environmental stresses under the controlled conditions of the laboratory (Walsh & Merrill, 1984). It is possible that in the field greater mucilage production could provide greater protection for the desmids against the plant litter-derived inhibitor(s). However, according to Coesel & Wardenaar (1994) copious mucilage production is likely only in those desmids growing in oligo-mesotrophic habitats, not eutrophic waters in which decomposed plant litter is likely to be used. Investigations using Indian ink to detect the presence of mucilage showed that all of the species tested had some mucilage surrounding their cells (results not shown). The desmids tested in this investigation have been shown to be susceptible to plant litter, suggesting that the type of mucilage is unlikely to provide any protection from the inhibitors, although further tests on a wider range of species would be valuable to establish more clearly the importance of the type and/or the amount of mucilage.

3.3 Division Chlorophyta

Class Chlorophyceae

3.3.1 Order Chlorococcales

In freshwater habitats species belonging to the order Chlorococcales are often abundant where nutrient concentrations are high (Hoek *et al.*, 1995). Growth occurs mainly during the early stages of thermal stratification or in the early stages of autumnal mixing, since some turbulence is required to keep the cells in suspension (Happey-Wood, 1988).

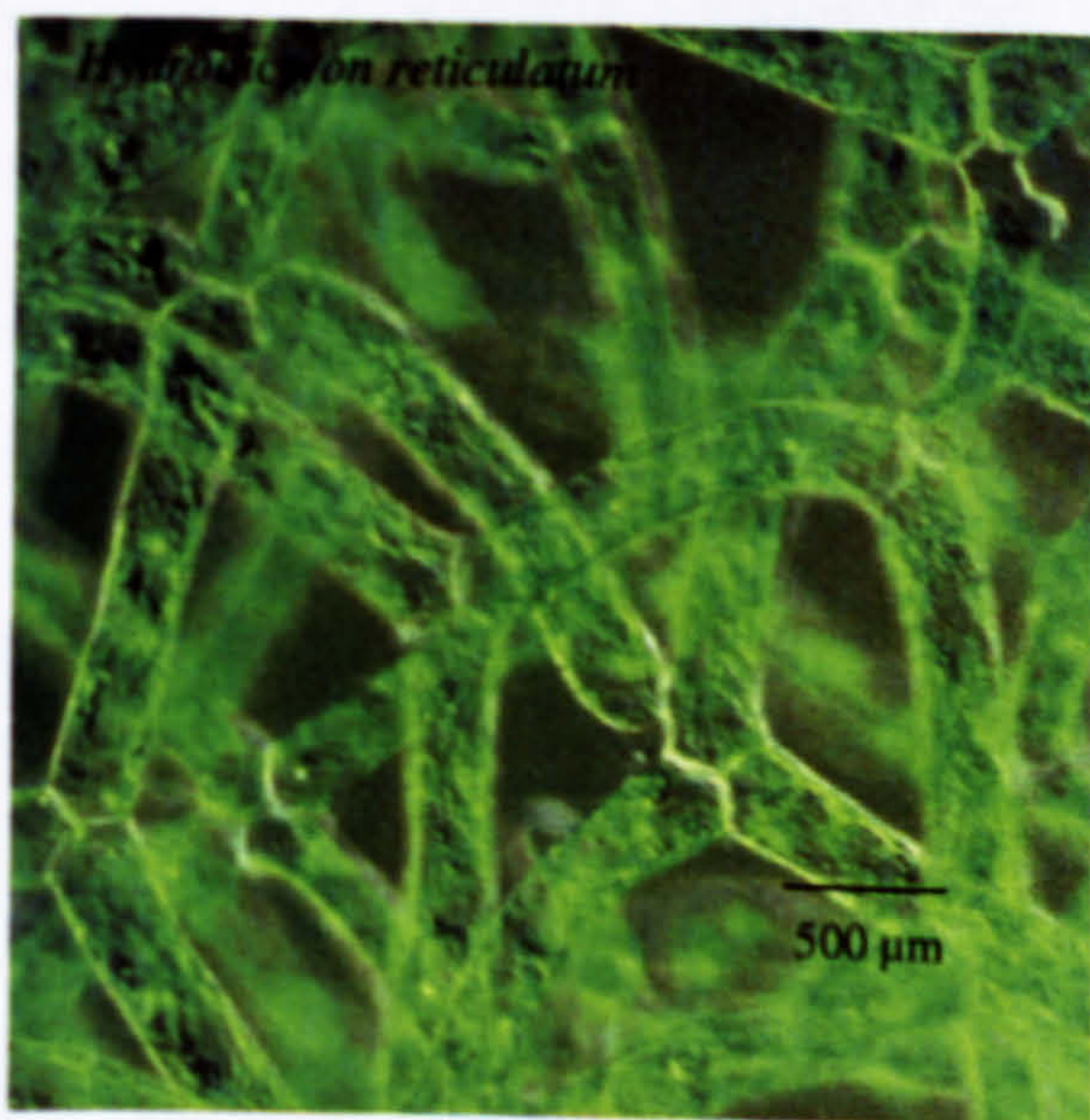
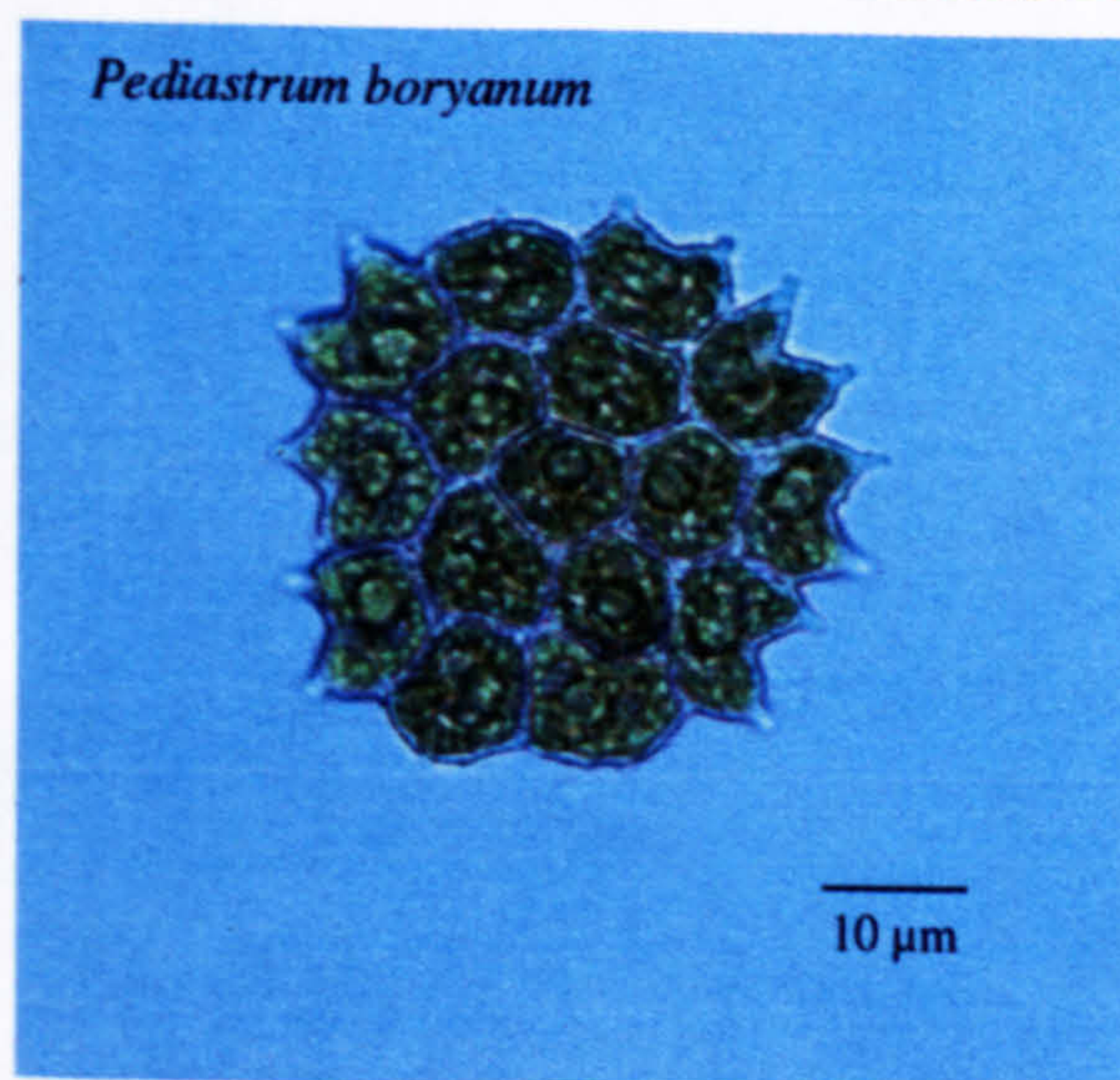
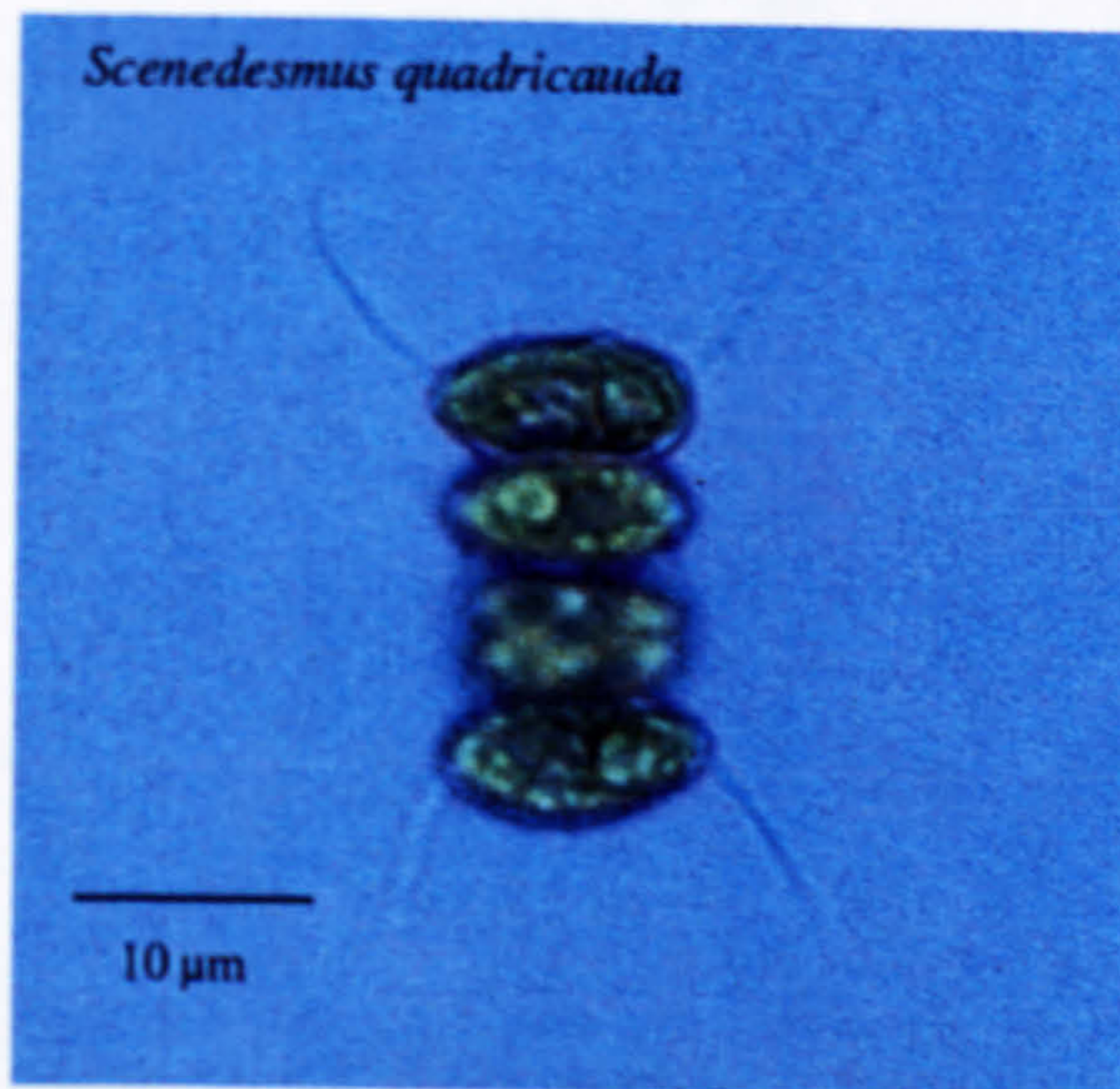
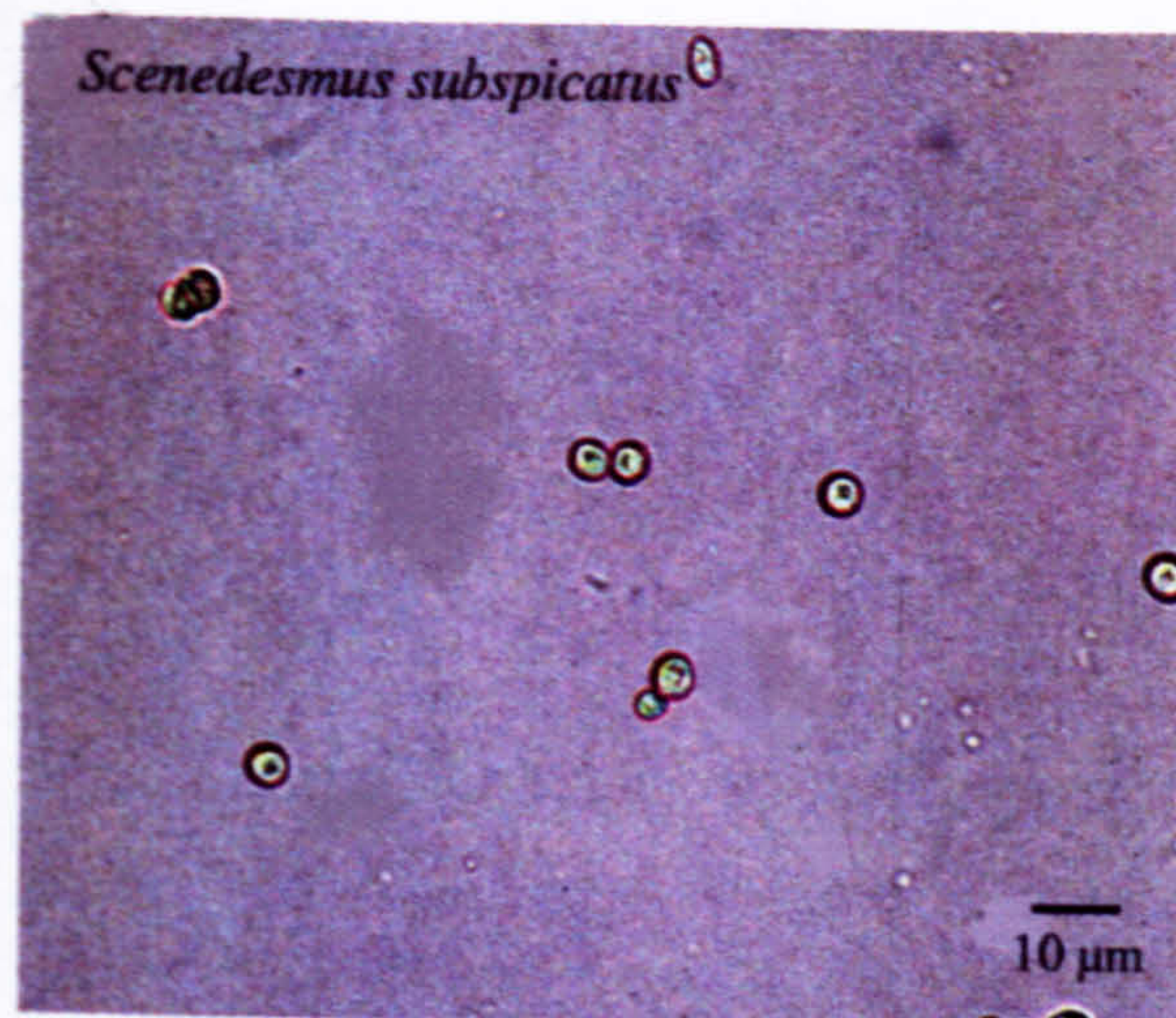
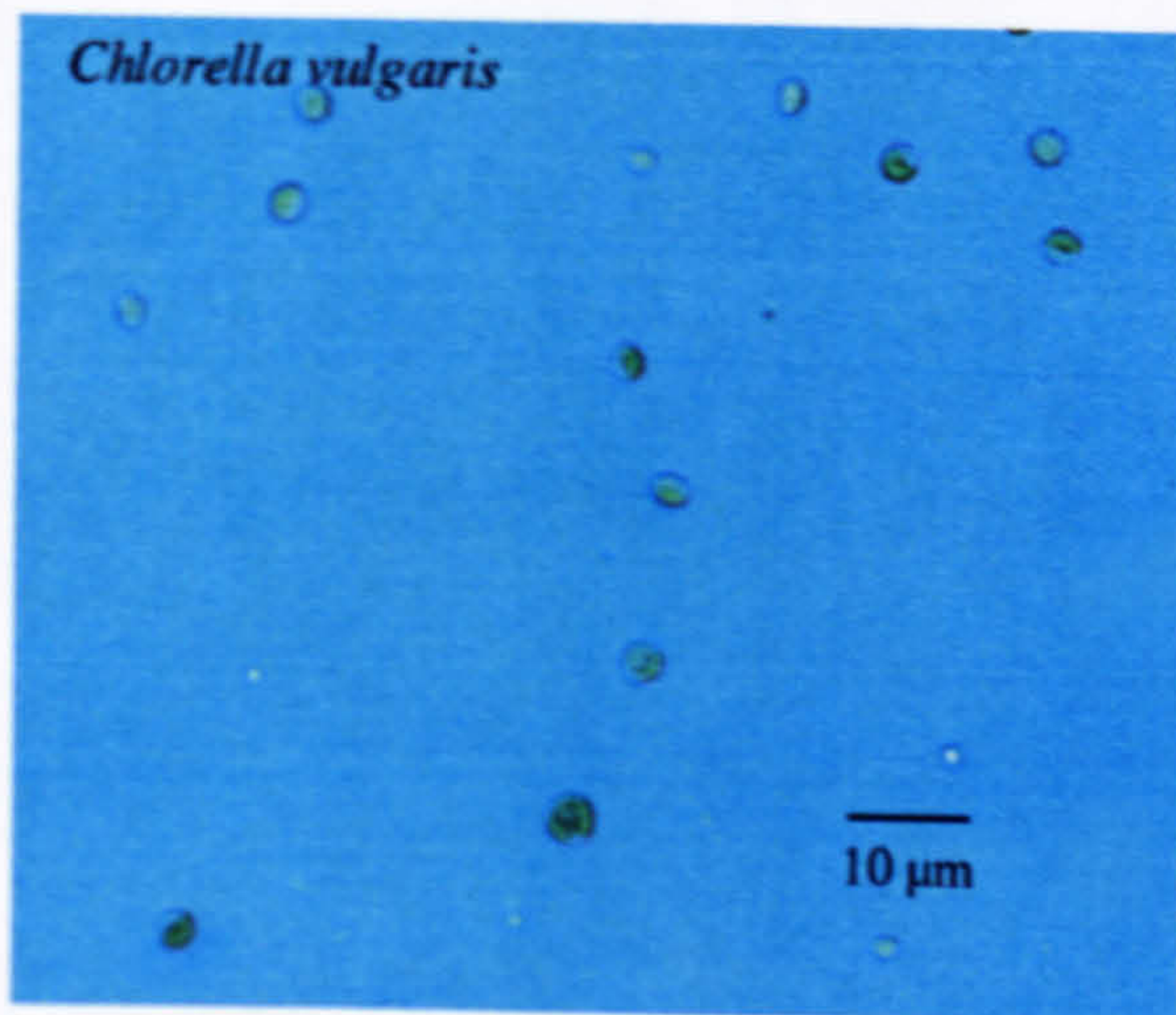


Plate 3.3 Members of the Chlorococcales, Chlorophyceae which were assayed.

Chlorella vulgaris

Chlorella vulgaris is a single-celled, coccoid alga (Plate 3.3) which reproduces by non-flagellate, asexual reproductive cells known as autospores. The number of autospores released by the mother cell is controlled either by internal or external conditions and usually 2,4,8 or 16 autospores are produced (Kuhl & Lorenzen, 1964). The cell wall contains a sporopollenin-like substance making it resistant to microbial attack (Hoek, 1995) and to non-oxidative chemical treatments (Derenne *et al.*, 1992). *Chlorella* is a common genus able to withstand high organic loading and is frequently found in sewage stabilization ponds (Palmer, 1980). *C. vulgaris* can grow planktonically and on soils and surfaces (Philipose, 1967; Hindák, 1984). Problems exist in *Chlorella* taxonomy since the genus appears to be genetically highly heterogeneous (Kessler, 1984; Hoek, 1995); consequently comparisons between members of the same genus have to be made with care.

Pillinger (1993) showed that decomposed barley straw inhibited *C. vulgaris* in laboratory cultures, but large amounts of straw were required to attain any degree of inhibition (up to 10 kg dry mass m⁻³ medium). The same strain was tested again to check the results of Pillinger (1993) and to see whether *C. vulgaris* was susceptible to oak leaf FPOM since previously only *Chlorella pyrenoidosa* had been tested with oak leaf (Ridge *et al.*, 1995).

Scenedesmus species are widely distributed throughout the world. Reproduction is most commonly asexual by means of autospores which are released from the mother cell to produce a new colony (coenobium). Daughter colonies may be morphologically identical to the parent or they can exhibit remarkable phenotypic plasticity, with environmental conditions defining different forms or ecomorphs of the species (Trainor, 1996). The cell wall consists of three layers, an inner layer of cellulose, a layer of sporopollenin-type material and an outer layer of pectin and/or mucilage (Soeder & Hegewald, 1988; Trainor,

1996). The wall is also often ornamented, which may play a part in reducing sinking rates and/or preventing grazing by small invertebrates (Hoek, 1995; Trainor, 1996) and, as with *C. vulgaris*, the sporopollenin layer is resistant to decay.

Scenedesmus subspicatus

S. subspicatus is a single-celled member of the *Scenedesmus* genus (Plate 3.3), although under different environmental conditions (such as varying temperature and the presence of *Daphnia*) coenobia may be produced (Trainor, 1993; Hessen & Vandonk, 1993). It is a planktonic genus, normally occurring in ponds and small lakes and is widely used in ecotoxicity testing (Tompkins *et al.*, 1995), where it has proved to be susceptible to a wide range of potential organic and inorganic water pollutants (Kühn & Pattard, 1990).

Scenedesmus quadricauda

(= *Scenedesmus communis*)

Scenedesmus quadricauda is a misapplied name since it has been given to many different species having spines arising from the apices of each marginal cell. Hegewald (1977) established the species *Scenedesmus communis* for the forms conforming to the original description. To avoid confusion I have used the term *Scenedesmus quadricauda* throughout this thesis since it is still commonly applied in the algal literature and is used by the culture collections from which my test species were obtained.

S. quadricauda is a colonial species (forming a coenobium) (Plate 3.3) commonly consisting of four cells, although this can vary depending on conditions to produce coenobia of 2, 4, 8 or 16 cells (Pentecost, 1984; Pickett-Heaps, 1975). Reproduction is asexual with complete daughter coenobia being formed within a single cell of the parent colony. *S. quadricauda* grows planktonically and/or associated with submerged surfaces, is commonly known to form blooms (Philipose, 1967) and can be widespread in areas of high organic enrichment, such as sewage stabilization ponds (Philipose, 1967; Palmer, 1969; Palmer 1980). It is also one of the most tolerant species of organic pollution ranked by Palmer (1969). Two different strains were used (see Table 2.5) to test for any possible intraspecies differences in susceptibility to plant litter derived inhibitor(s).

Hydrodictyon reticulatum

This is a colonial alga forming large cylindrical nets which can grow up to 1.1 metres long (Pocock, 1960). It is a well-known nuisance alga, often forming floating scums in eutrophic waters (Pocock, 1960; Pickett-Heaps, 1975; Hawes & Smith, 1993); it also has a relatively low requirement for nitrogen compared to other nuisance species (Hall & Payne 1997). The individual cells are multi-nucleate (coenocytic), cylindrical in shape and are attached to two other cells at each end giving the alga its characteristic net-like shape (Plate 3.3).

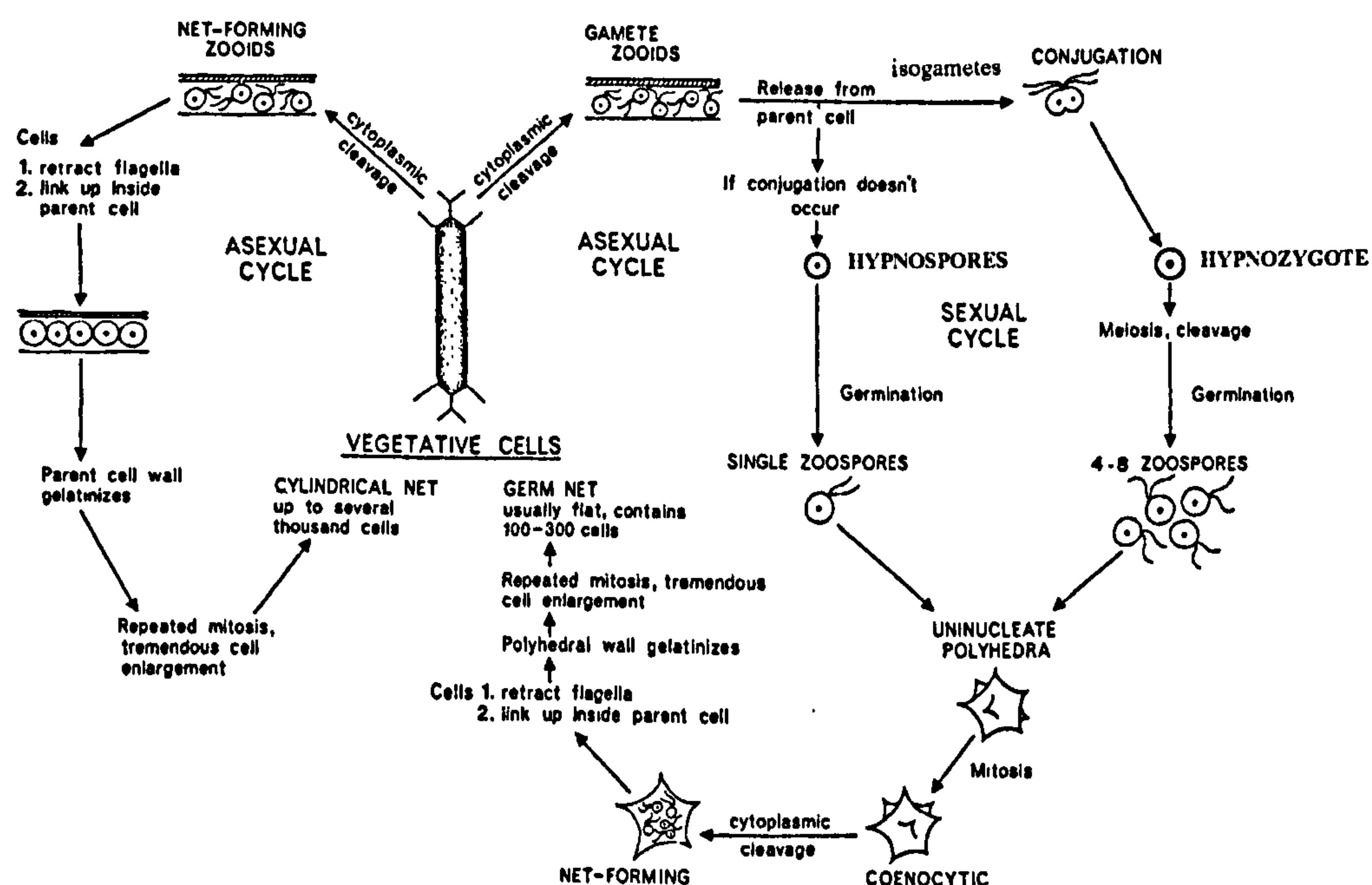


Figure 3.7 Life cycle of *Hydrodictyon reticulatum*. In this study only the asexual cycle (to the left of the figure) where zooids are retained within the parental cell wall is important. (Modified from Pickett-Heaps, 1975).

During asexual reproduction (Figure 3.7) cytoplasmic cleavage divides the cell into many biflagellate zooids, which are confined within the parental cell wall. The zooids aggregate and adhere to each other in a regular pattern, whereupon they start to expand and grow into the multinucleate cells which make up a whole new daughter net. The daughter net is liberated via disintegration of the parental cell wall and any further increase in cell

size is by expansion alone. Each parental cell is capable of producing a whole new net, which gives *H. reticulatum* a large potential for biomass increase.

Asexual reproduction can also be brought about by the direct release of the zooids, instead of them being assembled within the parental cell wall, or sexual reproduction can take place (Figure 3.7). However, none of these methods of reproduction was observed and they were not considered to be relevant to the present investigation (see below).

During asexual reproduction by 'net-forming zooids' (Figure 3.7) *H. reticulatum* has well-defined phases of cell division (within the parental cell) and cell expansion (after daughter net release). If susceptible to the inhibitor(s), *H. reticulatum* was thought to be an ideal test organism to try and find out which parts of the growth cycle (division or expansion) were affected by decomposed plant litter. Cell counting was not an option for estimating biomass due to the filamentous nature of the alga. Chlorophyll *a* analysis was also problematical with the low biomass present in the early stages of *H. reticulatum* growth, since the measurement of small amounts of chlorophyll *a* was impractical and inaccurate with the facilities available. It was considered more productive to focus on finding out whether *H. reticulatum* cells could divide and expand normally in the presence of the inhibitor(s). Cell dimensions were measured to investigate the expansion phase of the cell cycle and the production of new daughter nets was recorded, since their presence indicated that cell division had occurred.

Pediastrum boryanum

Pediastrum spp. are known to form blooms in water bodies ranging from the arctic to tropical and temperate regions, being particularly common in nutrient-rich freshwaters (Hoek *et al.*, 1995; Prescott, 1972; R. Croome, pers. comm.). *P. boryanum* is composed of a flat plate of, commonly, 16 or 32 cells (Philipose, 1967) with the outer cells usually bearing two horns or prongs (see plate 3.3). Silica is a component of the cell wall, though not in the quantities in which it is present in the diatoms (Millington & Gawlik, 1967;

Gawlik & Millington, 1969). *P. boryanum* also has an outer layer of a sporopollenin-like material making it resistant to decay (Gunnison & Alexander, 1975; Hoek *et al.*, 1995).

Asexual reproduction occurs largely as in *Hydrodictyon reticulatum* (see Figure 3.7) through the formation of biflagellate zoids that are discharged together into a vesicle extruded from the parental cell wall. The zoids arrange themselves within the vesicle, which is formed from the inner-most layer of the parental cell wall, to form a complete daughter colony (Davis, 1964; Millington *et al.*, 1981).

Sexual reproduction also resembles that of *H. reticulatum* (Figure 3.7). A resistant hypnozygote is formed from the fused isogametes, from which zoospores emerge on germination. Each zoospore develops into a polyhedra with subsequent germination and differentiation of the biflagellate zoids to form a new daughter colony.

As with *H. reticulatum*, *P. boryanum* has distinct cell division and cell expansion phases of growth. As a microscopic species *P. boryanum* was easier to assay and compare with the other microscopic species tested than with the filamentous *H. reticulatum*. *H. reticulatum* is generally thought to be characteristic of fairly pure waters (Philipose, 1967), although opinions do differ (Hall & Payne, 1997), whereas *P. boryanum* is characteristic of waters with medium organic content (Philipose, 1967). These contrasting preferences in water type may be displayed in their different abilities to survive in the presence of decomposed plant litter.

3.3.2 Results

Very high amounts of plant litter were required to inhibit the growth of *Chlorella vulgaris*. From Figure 3.8 it can be seen that 50% inhibition was still not obtained with a dose exceeding 5400 g dry mass of barley straw m⁻³. A higher mass of straw was not used since shading may then have played a part in reducing cell numbers. From Table 3.2 (page 86) it

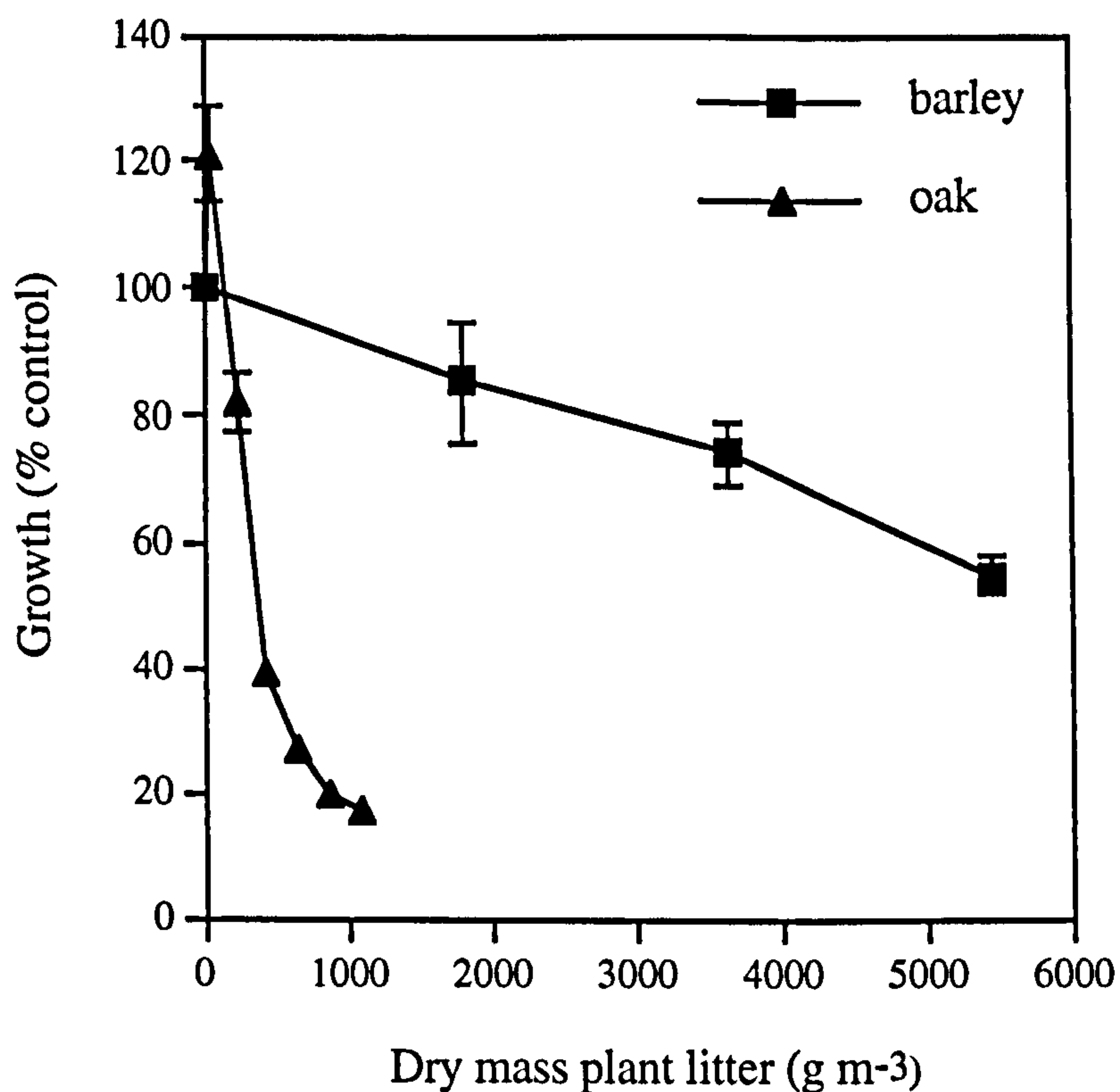


Figure 3.8 Growth of *Chlorella vulgaris* with different masses of oak leaf FPOM and barley straw. Values shown are means ($n=5$) \pm SE. Inhibition is significant ($p < 0.001$).

can be seen that of the species found to be susceptible to plant litter *C. vulgaris* appears to be the least sensitive, requiring the most oak leaf FPOM or barley straw to obtain 50% inhibition in growth (or nearly 50% inhibition for barley straw).

Figure 3.9 clearly shows that *Scenedesmus subspicatus* was not inhibited at all, with growth actually increasing to over 200% of the control value with barley and 150% with oak leaf FPOM. It appears that at higher doses of straw, although still stimulated, the straw seemed to be showing some effect and the stimulation decreased. Further experiments using over 3600 g dry mass straw m⁻³ medium still showed stimulation of over 200% of the control value (results not shown) suggesting that the original decrease was due to variation in the decomposed straw used in the assay.

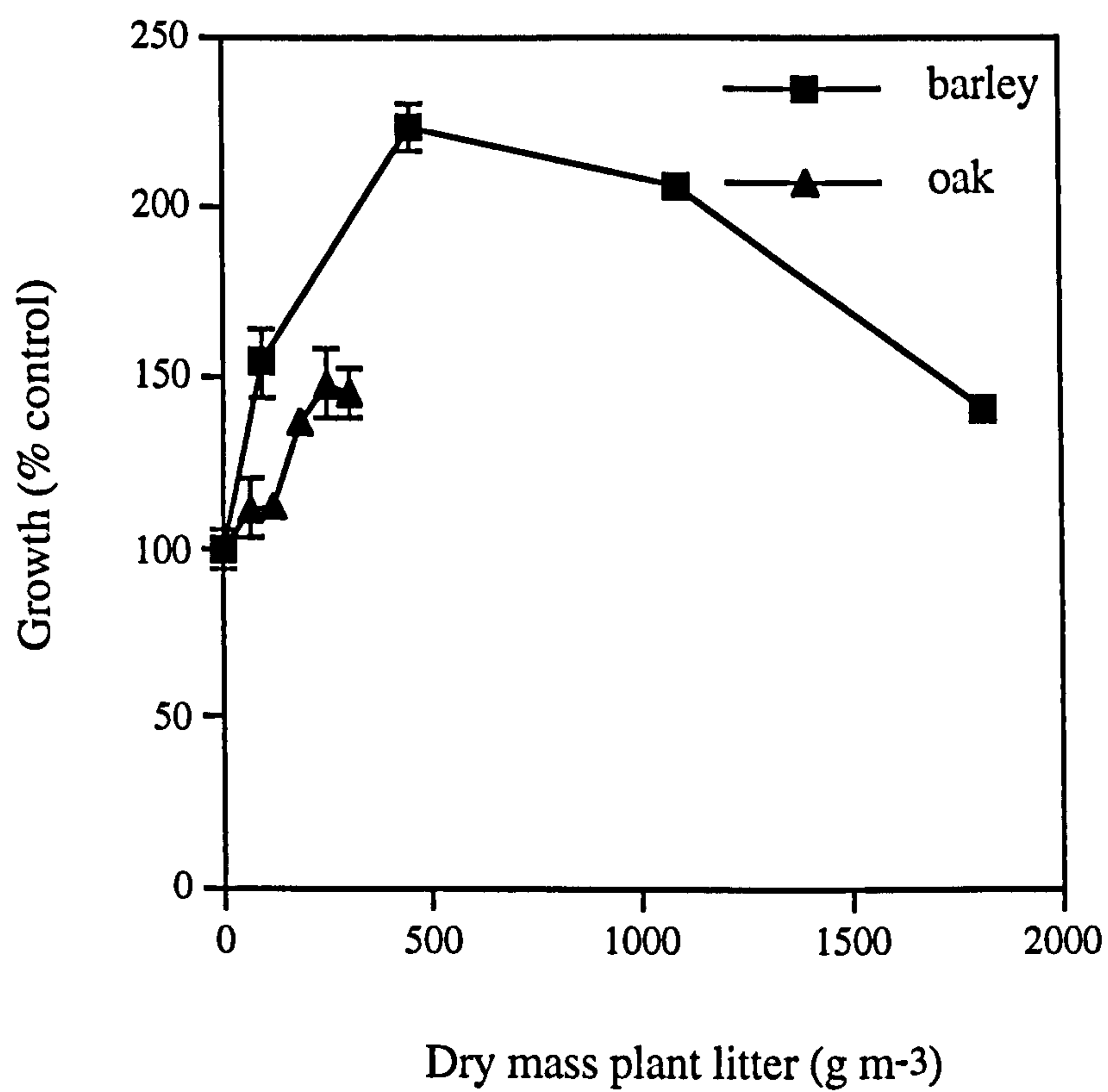


Figure 3.9 Growth of *Scenedesmus subspicatus* with different masses of oak leaf FPOM and barley straw. Values shown are means (n=5) \pm SE. Stimulation is significant in both cases ($p < 0.001$).

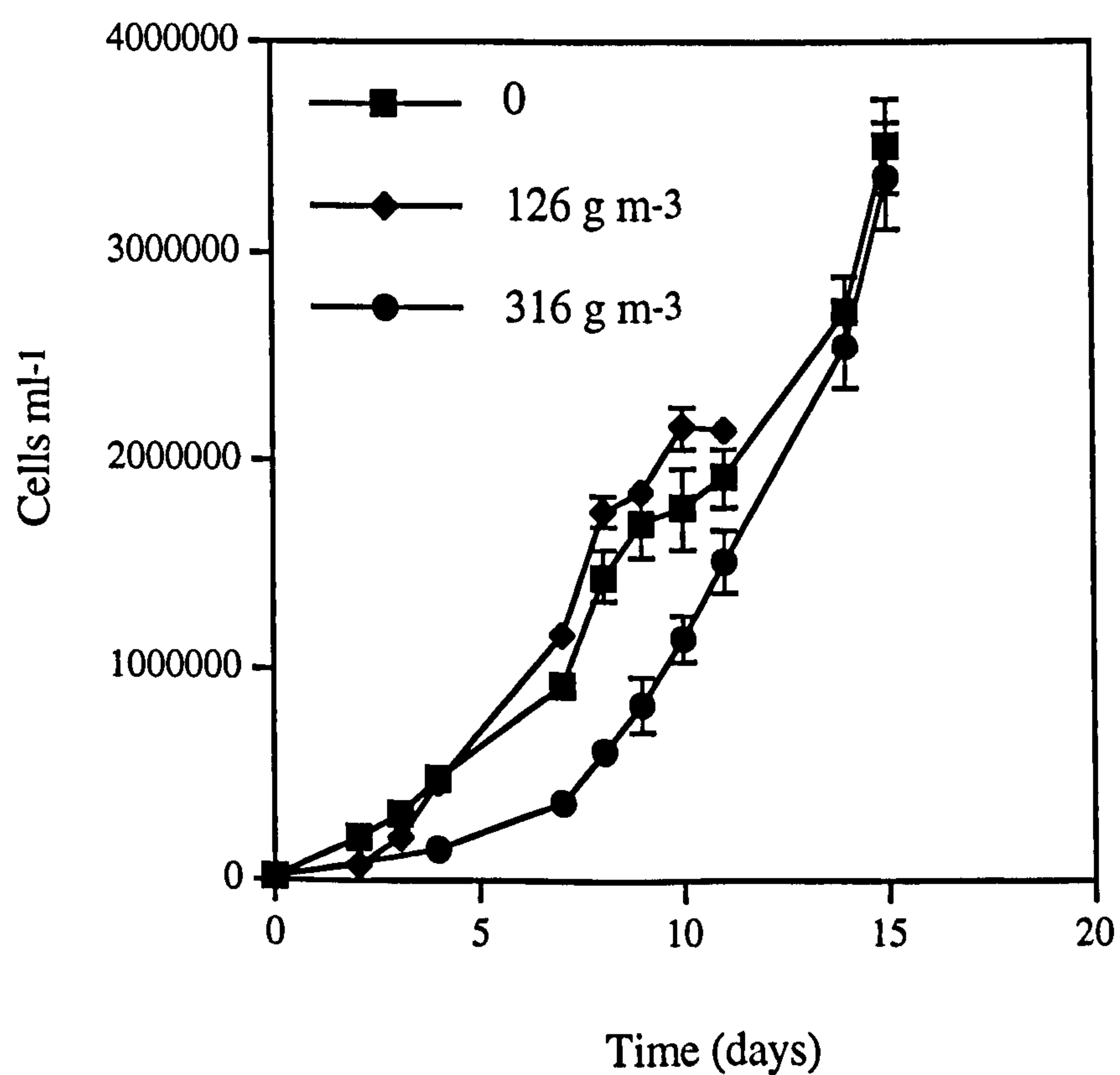


Figure 3.10 Growth of *Scenedesmus quadricauda* CCAP 276/21 over time with different amounts of oak leaf FPOM (g dry mass m⁻³ medium). Values shown are means (n=5) \pm SE.

A two-way repeated measures ANOVA of *Scenedesmus quadricauda* with oak leaf FPOM showed a significant ($p < 0.05$) interaction between time and the mass of oak leaf FPOM, meaning analysis of differences at specific time points was not valid. The presence of an interaction implies differences in the shapes of the curves, although no probability can be attributed to the differences. From Figure 3.10 there appears to be an initial inhibition in growth of *Scenedesmus quadricauda* with the highest amount of oak leaf FPOM (316 g m^{-3}) and a recovery after about eleven days, whereupon cell numbers were similar to control values. With 126 g m^{-3} of oak leaf FPOM there was no overall inhibition, although there appeared to be a slight inhibition at days two and three.

The results for *Scenedesmus quadricauda* CCAP 276/21 and *S. quadricauda* Sciento with barley straw were very variable and some examples of assays are shown in Figure 3.11. Figure 3.11 (a) & (b) show no significant differences between cell numbers in barley treated cultures and control cultures. Figure 3.11(c) shows a significant ($p < 0.05$) interaction between time and barley straw suggesting that there are differences in the shapes of the curves which, from their shape, implies that *Scenedesmus quadricauda* is inhibited in the early stages of growth. In Figure 3.11(d) there is a significant ($p < 0.001$, student's t-test) inhibition of growth after two days but cell numbers are not significantly different from the control after five days. Overall no differences between the Sciento and CCAP 276/21 strains can be ascertained from the present data. A member of the same genus, *Scenedesmus subspicatus* showed different results to *S. quadricauda*. Growth was always shown to be stimulated (Figure 3.9) and no initial inhibition of growth was ever observed with *S. subspicatus*.

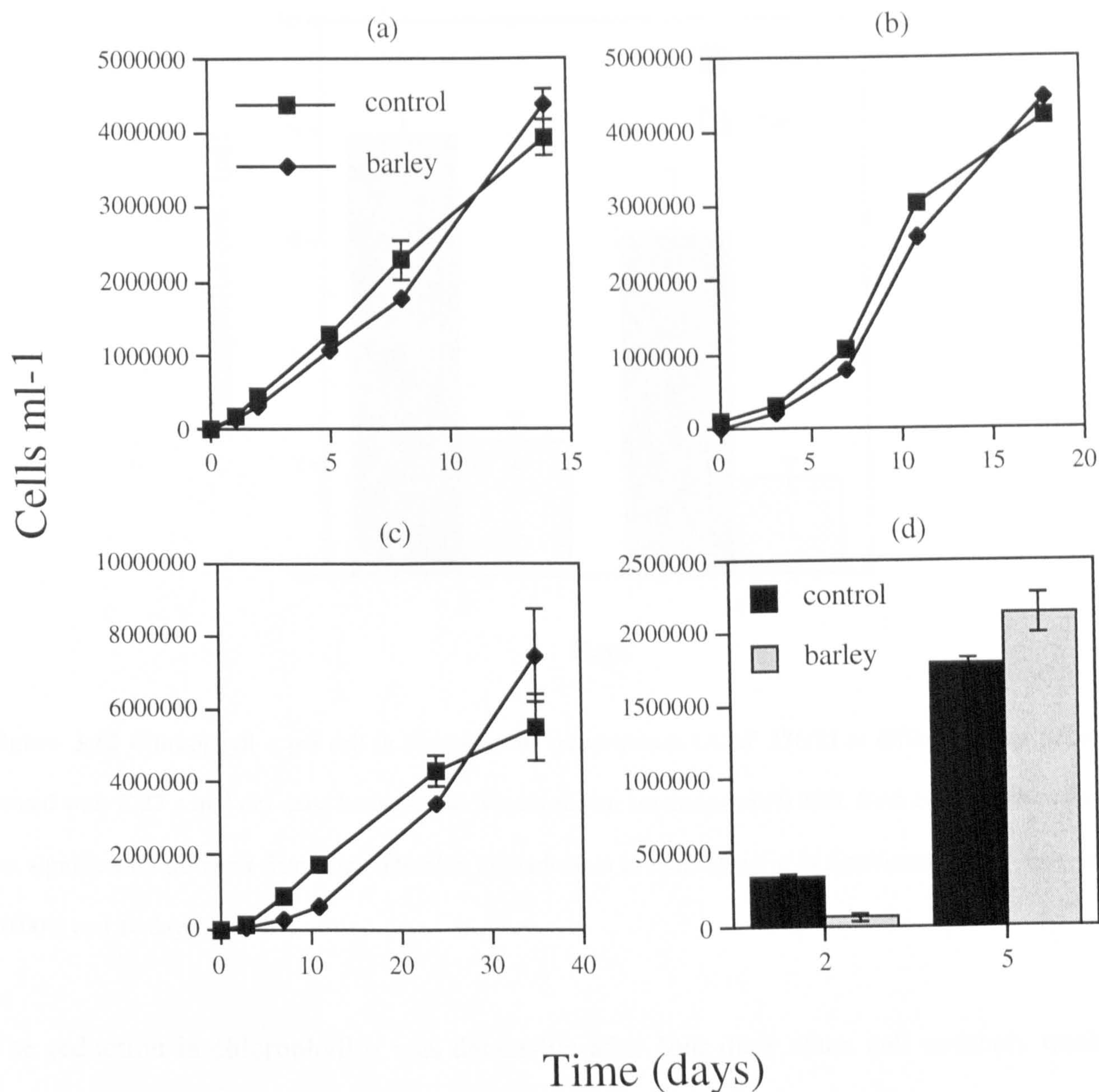


Figure 3.11 The growth of (a) & (c) *Scenedesmus quadricauda* (Sciento) and (b) & (d) *Scenedesmus quadricauda* CCAP 276/21 over time with 2727 g m⁻³ dry mass of barley straw. Values shown are means (n=5) \pm SE.

During the assays it was observed that the cultures of *Scenedesmus quadricauda* appeared to be markedly less green when grown in the presence of plant litter. Figure 3.12 shows that chlorophyll *a* levels in each cell were significantly reduced when grown in the presence of barley straw.

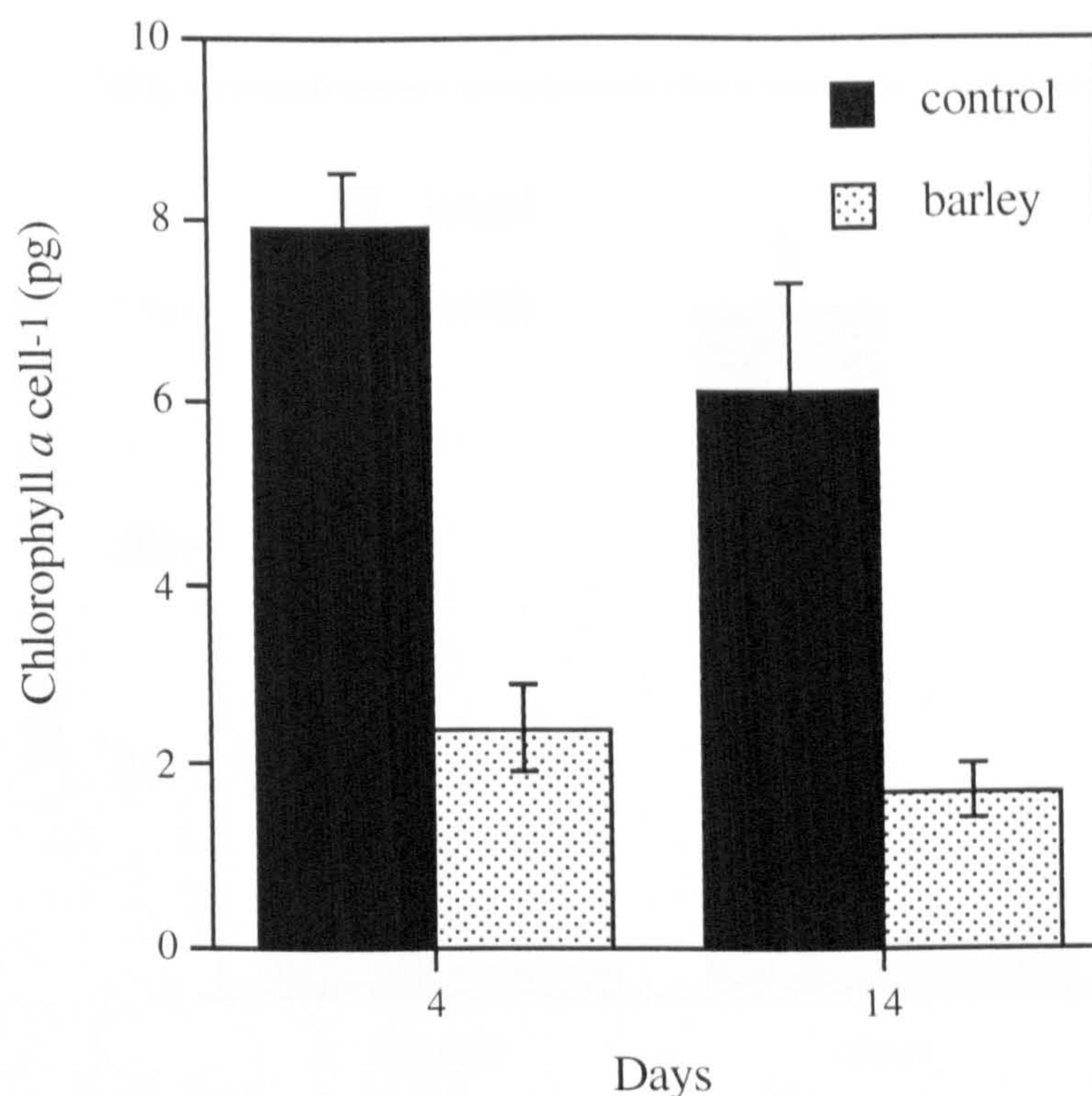


Figure 3.12 Chlorophyll *a* per cell in *Scenedesmus quadricauda* CCAP 276/21 at different times when treated with 2727 g m⁻³ dry mass barley straw. Values shown are means (n=5) \pm SE. Both control values are not significantly different from each other but the reduction in chlorophyll *a* is significant after 4 days ($p < 0.001$) and 14 days ($p < 0.01$).

The reduction in chlorophyll *a* was detectable after four days when cell numbers were inhibited by 24% of control values and after fourteen days when the cell numbers had recovered and were not significantly different from the control values (further investigations are shown in section 4.4). Measurement of the cell area using computer aided techniques (see section 4.2.2) showed a small but significant increase in cell area in the barley treated cultures, as seen in the plane of the microscope ($P < 0.05$, $n=50$). No replicate measurements were made but cell size was not observed to be reduced in any of the other assays carried out with *S. quadricauda*.

Results for Hydrodictiaceae

Figure 3.13 clearly shows that cell expansion in *Hydrodictyon reticulatum* was greatly increased in the presence of decomposed barley straw. From Table 3.1 daughter net formation did not appear to be affected even after three 'generations' in the presence of

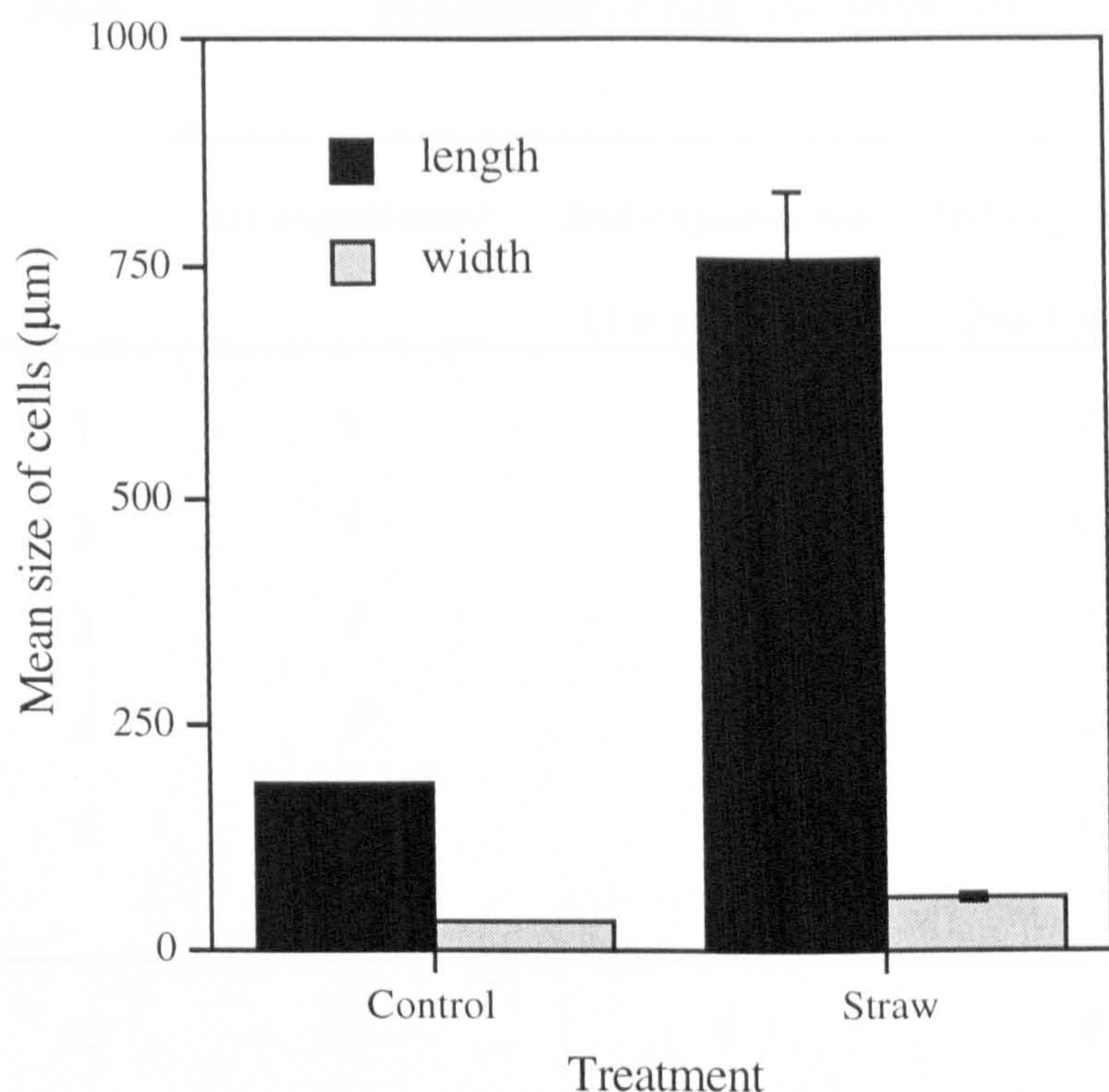


Figure 3.13 Length and width of *Hydrodictyon reticulatum* cells when grown with and without decomposed barley straw (2700 g dry mass m⁻³ medium). Values shown are means of four replicate flasks and 80 measured cells. Error bars show standard error (where not shown errors are too small to appear). Students t-test shows increases in length and width are significant $p < 0.001$.

Footnote: For this assay small daughter nets which had just been released from the parental cell were placed into the different treatments and the cell dimensions measured after ten days growth (width was measure at the centre of each cell).

decomposed straw, although biomass production was not measured. The evidence suggests that *H. reticulatum* is resistant to decomposed barley straw at the mass of straw used in these assays (2700 g dry mass m⁻³ medium). The production of new daughter nets also appeared to be unaffected in unreplicated experiments using over 5000 g dry mass straw m⁻³ medium.

Treatment	Flask	Production of new 'daughter nets'		
		1st experiment	2nd experiment	3rd experiment
			(1st transfer)	(2nd transfer)
control	1	√	√	√
	2	√	√	√
	3	√	√	√
	4	√	X	-
	5	√	√	√
barley	6	√	√	√
	7	√	√	√
	8	√	√	X
	9	√	√	√
	10	√	√	√

Table 3.1 The asexual growth of new daughter nets of *Hydrodictyon reticulatum* when grown with and without barley straw (2700 g dry mass m⁻³ medium). √ = production of new nets, X = no production of new nets.

Footnote: A single net of *H. reticulatum* was placed in each flask and the production of new nets was noted. On the production of new nets, a randomly chosen 'new daughter net' was placed into a new flask of the treatment required and the process repeated. In effect the nets were in the presence of the treatment for three 'generations'.

Pediastrum boryanum was susceptible to both oak leaf FPOM and barley straw (Figure 3.14), although the amount of plant litter required to achieve a 50% inhibition of growth was relatively high compared to most of the other species which were found to be susceptible (see Table 3.2 page 86). Further investigations into the effects of plant litter on *P. boryanum* are described in Chapter 4.

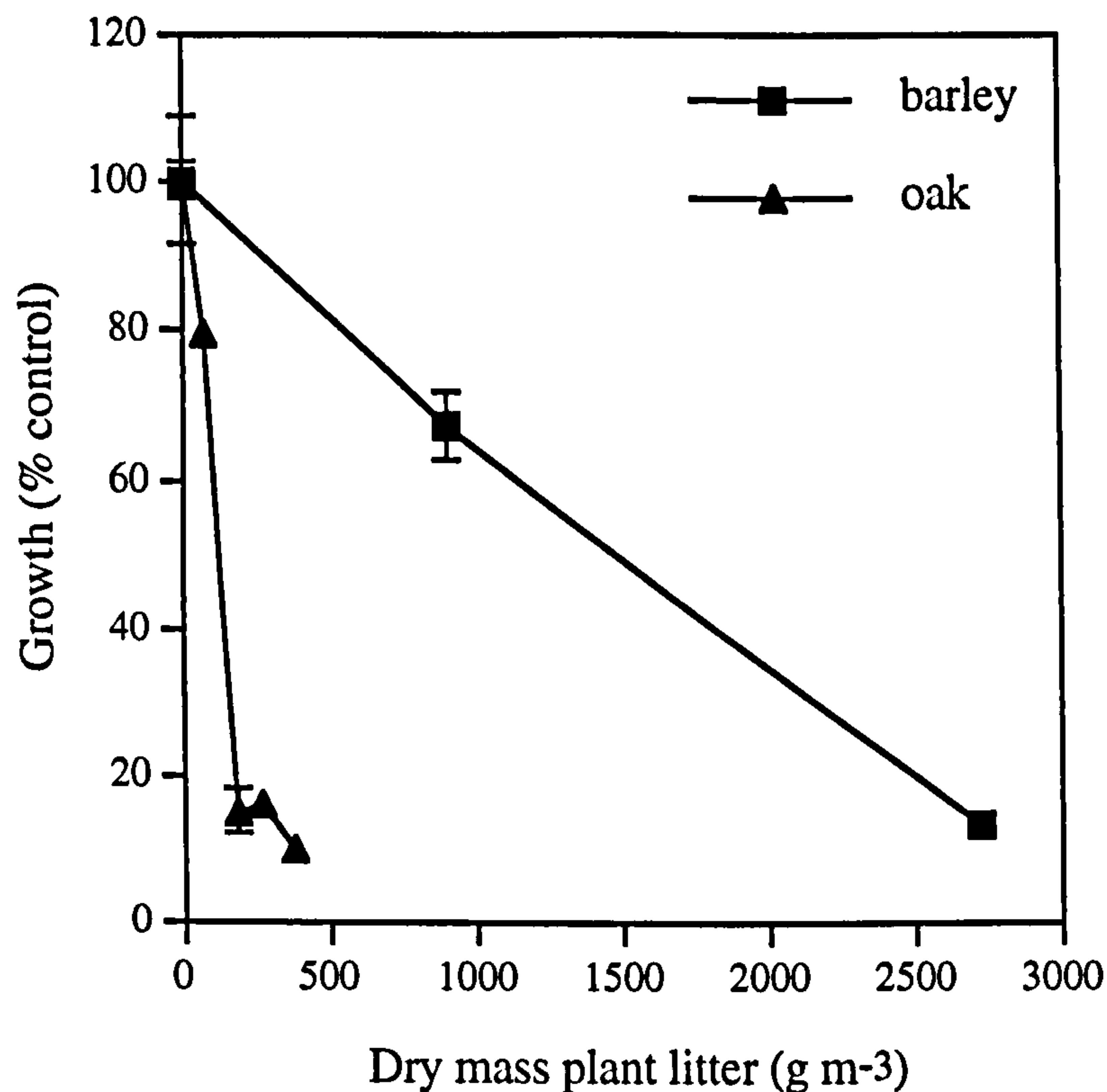


Figure 3.14 Growth of *Pediastrum boryanum* with different masses of oak leaf FPOM and barley straw. Values shown are means ($n=5$) \pm SE. Inhibition is significant $p < 0.001$.

3.3.3 Discussion

The results for *C. vulgaris* with barley straw correspond with those of Pillinger (1993) and those of Gibson *et al.* (1990) (who used a different strain of *C. vulgaris*) since a large mass of barley straw is required for inhibition (Figure 3.8). The amount of oak leaf required for inhibition is also comparable to the amounts required for inhibition in *Chlorella pyrenoidosa* (Ridge *et al.*, 1995), where with 1000 g dry mass m⁻³ of oak leaf FPOM growth was 10-50% of control values, depending on the length of decomposition time.

The results for *Scenedesmus quadricauda* are more variable with both types of plant litter. Growth was either not inhibited (Figure 3.11 (a) & (b)) or it was suppressed in the early stages of the assay, before cell numbers recovered to equal those of the control (Figures 3.10, 3.11 (c) & (d)). Why there should be so much variation with both oak leaf and barley straw is not known. A number of possible explanations might account for the initial inhibition then recovery of cell numbers. Walsh & Merrill (1984) suggested that

long-term assays introduce inaccuracies since the toxicants may degrade, adsorb to the flask walls, or a large ratio of algal biomass:toxicant may be obtained which can reduce the effects of the toxin. As distinct from the use of single additions of substances usually employed in ecotoxicity tests, plant litter is continually producing the inhibitor(s) via microbially-mediated release. The continuous release of inhibitor(s) should offset any toxin degradation or adsorption making these unlikely explanations for the recovery in cell numbers seen in *S. quadricauda*. The fact that *S. quadricauda* can recover after only two-five days growth (Figures 3.10 & 3.11) also suggests that the biomass to toxicant ratio effect is not acting in this case, but further assays are required.

The recovery in cell number and variation in the *S. quadricauda* assays is more likely to be due to the reaction of the organism itself to the toxin(s). This could be via the induction of protective mechanisms, such as detoxification of the inhibitor(s), active expulsion of the inhibitor(s) after having entered the cell or the complexation of the inhibitor(s) by algal exudates. It is known that *S. quadricauda* does exude increased amounts of protein and carbohydrates when stressed by phenolic and other organic compounds (see Marsálek & Rojícková, 1996), but whether this can act to overcome the toxic effects of litter-derived inhibitor(s) is not known. The possibility also exists that the recovery in cell numbers is not induced in the entire population of the initial inoculum, but is due to the growth of a resistant sub-population of cells present in the inoculum. Attempts were made to grow cells previously exposed to plant litter with fresh plant litter to see if growth differed compared to those not previously exposed. However, contaminant species which were already present on the litter (the plant litter was not sterilized before decomposition) and which reproduced during the time-course of the first assay made the results inconclusive, since unialgal inocula could not be obtained.

Conclusions

As a group the chlorococcalean species appear to be the most resistant of all the species tested. Most of the Chlorococcales were either completely resistant or only slightly susceptible to barley straw. The most susceptible of the chlorococcalean species was

Pediastrum boryanum and even this required quite a large amount of barley straw to produce 50% inhibition of growth (see Table 3.2 page 86).

The presence of a sporopollenin-like material in the cell wall might play some part in their resistance since both the *Scenedesmus* species (Hoek *et al.*, 1995; Trainor, 1996), *Chlorella vulgaris* (Hoek *et al.*, 1995) and *Pediastrum boryanum* (Gunnison & Alexander, 1975) have it in their cell wall. However, *Hydrodictyon reticulatum*, which appears to be resistant (see Figure 3.13 & Table 3.1), does not have this type of cell wall.

The only characteristic property common to all of the chlorococcalean algae tested is their method of reproduction. When reproducing asexually all of the algae produce daughter cells within the mother cell wall and so they are never directly exposed to the outside environment. In *C. vulgaris*, *Scenedesmus* spp. and *H. reticulatum* the daughter colonies are formed wholly within the mother cell wall before their release. In *Pediastrum boryanum* the zoospores, which go on to develop into a new daughter colony, are released into a vesicle produced only from the inner-wall layer of the parental cell (Millington *et al.*, 1981). The production of new daughter colonies in a vesicle and not within the entire mother cell wall could account for the relatively low resistance of *Pediastrum boryanum* compared to the other chlorococcalean algae tested. However, in *P. boryanum* mitosis occurs wholly within the mother cell wall (Millington & Gawlik, 1975; Millington *et al.*, 1981). If the confinement of reproduction within the mother cell wall did confer resistance upon the chlorococcalean algae, *P. boryanum* zoospores should be produced by mitosis. The release of zoospores into vesicles (i.e. outside the protection of the entire mother cell wall) would prevent them from developing further into new daughter colonies. These zoospores, confined within vesicles, should have been abundant in treated cultures but, in reality, numerous undeveloped colonies within vesicles were not observed.

3.4 Division Chlorophyta

Class Klebsormidiophyceae

3.4.1 Order Klebsormidiales (=Ulotrichales)

Family Ulotrichaceae

Stichococcus bacillaris

Stichococcus bacillaris has cylindrical cells arranged in very short filaments which tend to dissociate easily, so cells often occur singly (Plate 3.4). This species is commonly found in sub-aerial habitats on walls and trees and often occurs on fertile soils, although it can be found on surfaces in freshwater (Prescott, 1969, 1972, Pentecost, 1984). Since *S. bacillaris* occurs on terrestrial surfaces, it is prone to stresses such as desiccation and excessive light. It is also known to be able to survive and grow in artificial sea water of up to 300% strength (Ahmad & Hellebust, 1993). The ability to tolerate stress may be a factor in the resistance of algae to decomposed plant litter, consequently, *S. bacillaris* was thought to be a suitable species to test.

3.4.2 Results

Stichococcus bacillaris was inhibited by both barley straw and oak leaf FPOM. It can be clearly seen from Figure 3.15 that with lower masses of barley straw there was a stimulation of growth (see section 3.7.3), but with higher masses of straw (over 1000 g dry mass m⁻³) *S. bacillaris* was inhibited. When compared with other members of the Chlorophyta (Table 3.2 page 86), *S. bacillaris* appears to be one of the least susceptible species tested with only *Chlorella vulgaris* requiring more barley straw to produce 50% inhibition in growth, although relatively little oak leaf FPOM is required to obtain 50% inhibition in growth.

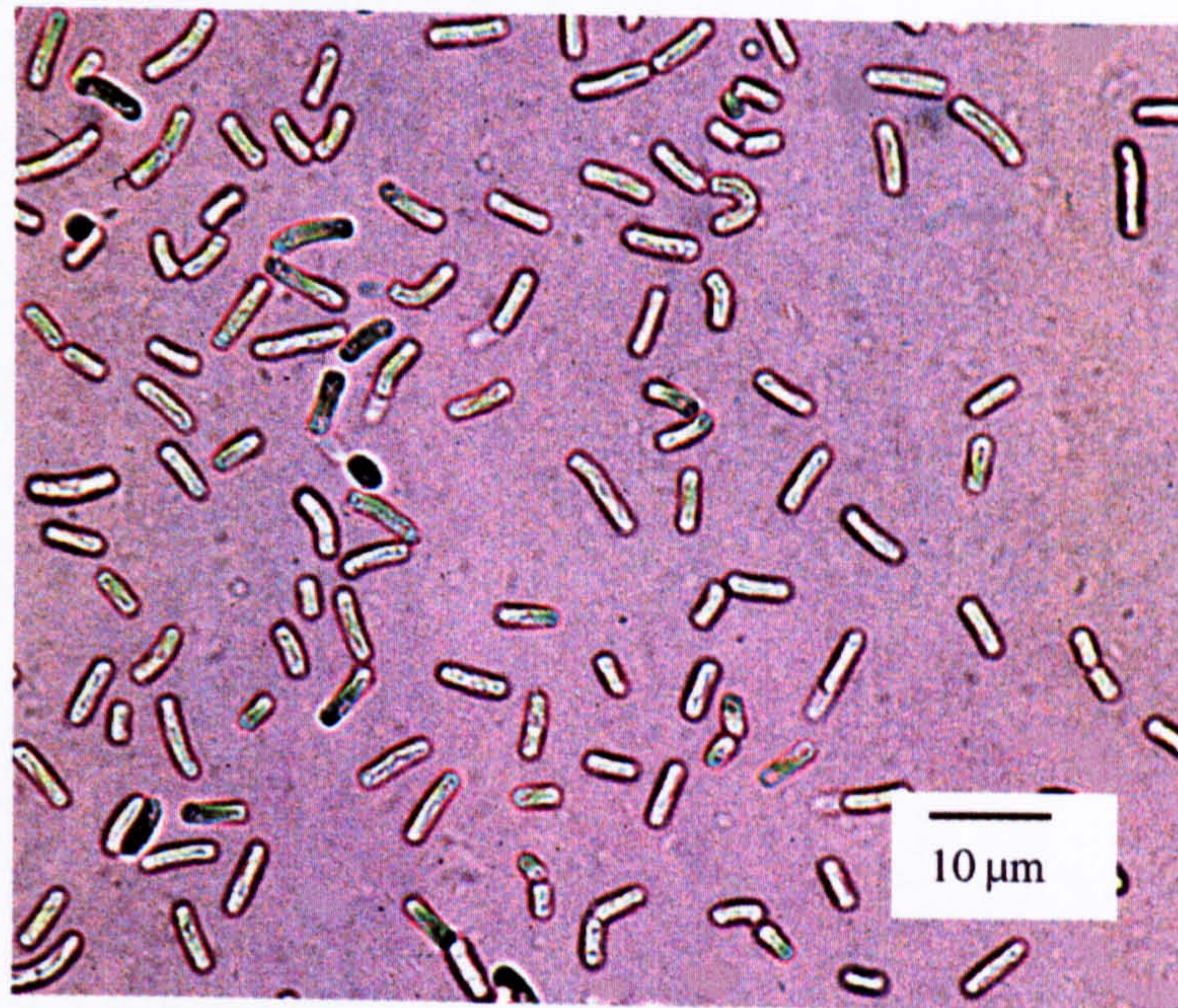


Plate 3.4 *Stichococcus bacillaris*

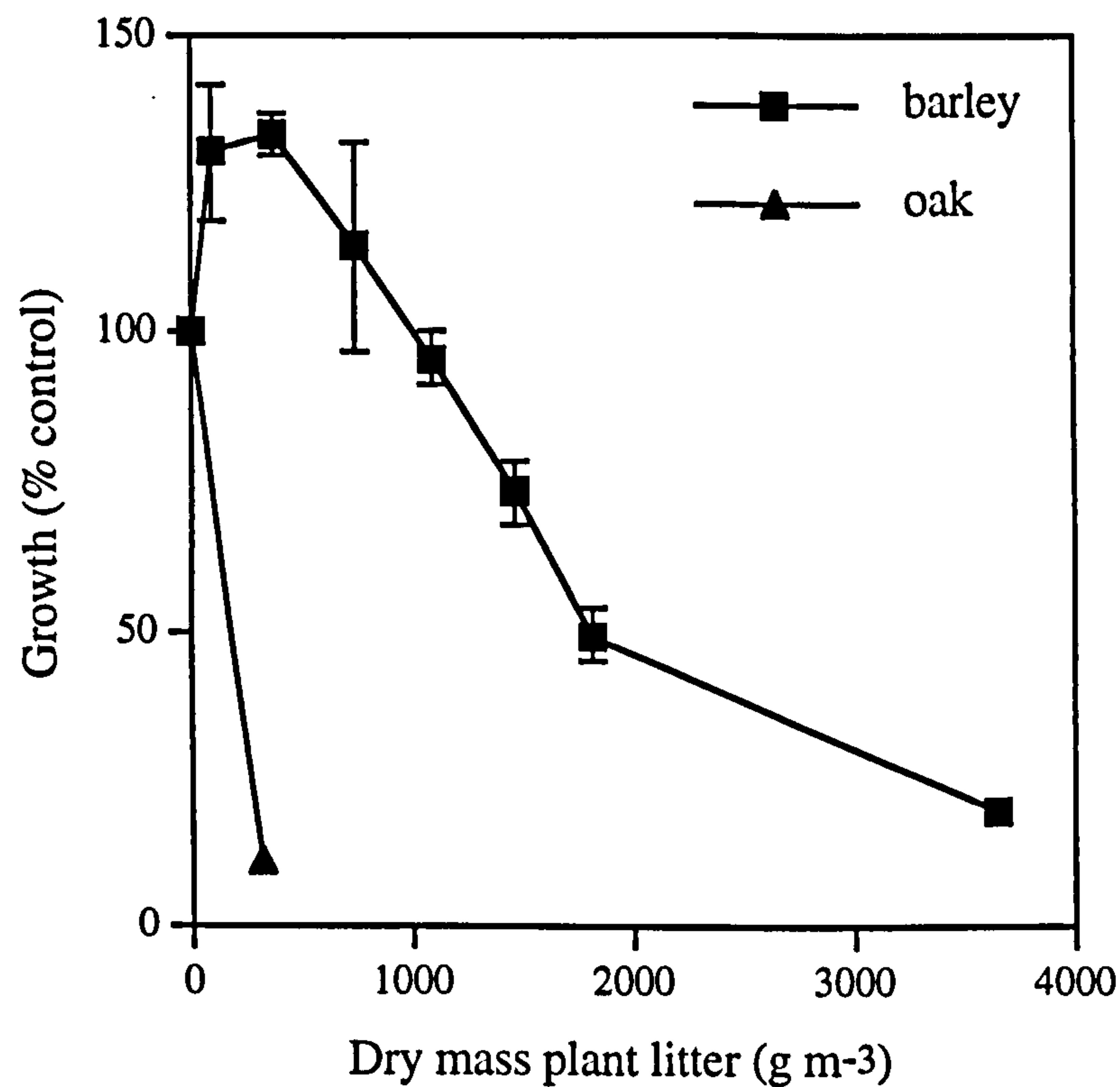


Figure 3.15 Growth of *Stichococcus bacillaris* with different masses of oak leaf FPOM and barley straw. Values shown are means (n=5) \pm SE. Inhibition is significant $p < 0.0001$.

3.5 Division Euglenophyta

Class Euglenophyceae

Euglenophytes do not possess a true cell wall, instead the outer plasma membrane is modified (Prescott, 1969). Strips of mainly proteinaceous material lie immediately beneath the plasma membrane, within the cytoplasm, to form a pellicle which encloses the whole cell (Dubreuil *et al.*, 1992; Hoek *et al.*, 1995). These strips are wound helically around the cell and they overlap and articulate allowing movement of the whole pellicle (Dubreuil *et al.*, 1992). No sexual reproduction has been observed and asexual reproduction occurs via mitosis and division of the cell in the longitudinal plane. Members of this division most commonly grow in small pools and ditches that have been organically enriched (Pentecost, 1984; Hoek *et al.*, 1995).

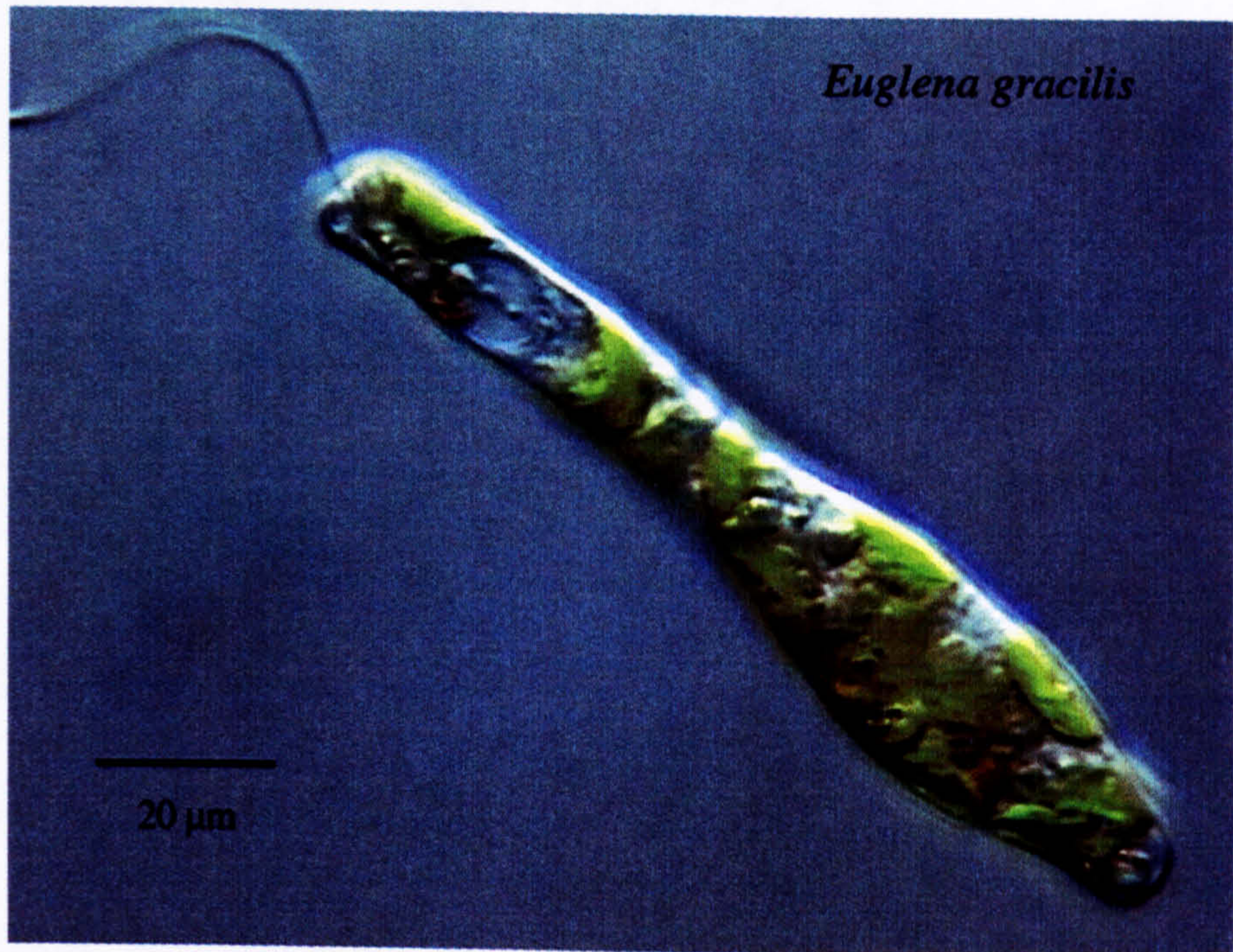


Plate 3.5 *Euglena gracilis*

3.5.1 Order Euglenales

Euglena gracilis

Euglena gracilis (Plate 3.5) is a unicellular species possessing two flagella. It is tolerant of high organic pollution (Palmer, 1969) and is often found in hypereutrophic waters, especially small pools (Reynolds, 1984a; Pentecost, 1984). As with many other *Euglena* species, *E. gracilis* can be grown heterotrophically in the dark, allowing assays to be carried out without the influence of light. It is possible that light could transform the organic matter dissolved in the medium (Lindell *et al.*, 1996; Bushaw *et al.*, 1996) and it has been proposed that this may produce free radicals which could inhibit the growth of algae (Barrett, 1994). However, assays in the dark using *Saprolegnia* 'fungi' (formerly classified as a fungus but now regarded as belonging to the class Oomycetes in the division Heterokontophyta) have shown barley straw still to be inhibitory (Cooper *et al.*, 1997). The susceptibility to decomposed plant litter of known photoheterotrophic organisms had not been investigated or compared with purely phototrophic organisms. It was considered prudent to test this since an inherent ability to assimilate organic matter may confer resistance upon an alga.

3.5.2 Results

After three days growth in the presence of barley straw there was a significant inhibition of growth in *E. gracilis* when grown in the dark, whereas in the light no significant inhibition was found (Figure 3.16). After six days growth inhibition was found in both the light and dark grown cultures, but the dark grown culture was relatively more inhibited. The greater inhibition in the dark grown cultures was found in three replicate experiments. Continuation of the assays for a longer period of time was not possible due to dense growths of microbial contaminants which resulted from using an organic growth medium.

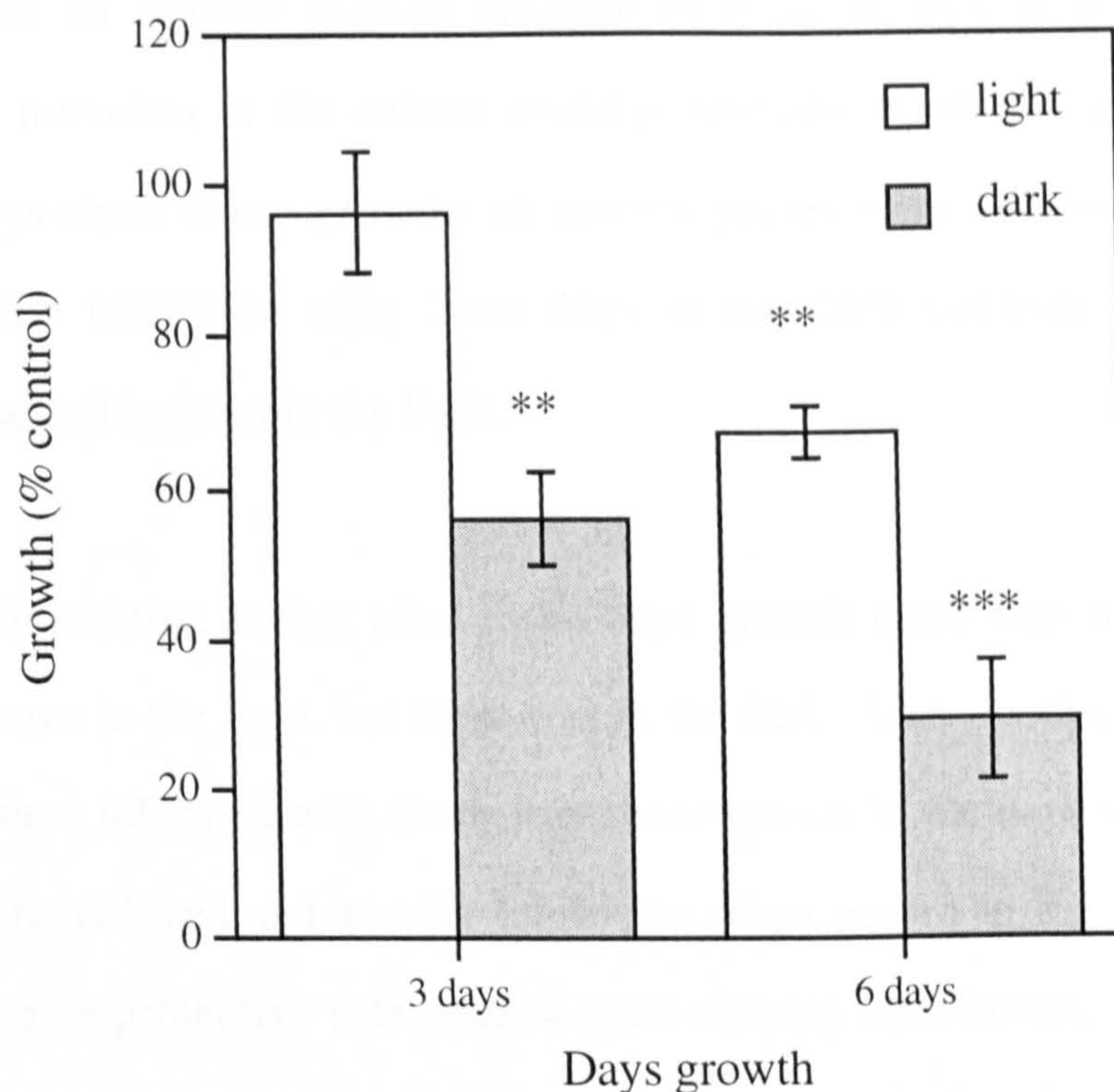


Figure 3.16 Growth of *Euglena gracilis* with barley straw. Values are means \pm SE (n=5). (Asterisks denote significant differences from the control (100% growth): ** p < 0.01, *** p < 0.001).

Footnote: Values for light assays are compared to a value for controls grown in the light and values for dark assays are compared to a value for controls grown in the dark. 2727 g dry mass m⁻³ of barley straw used in all assays.

3.5.3 Discussion

The fact that inhibition of *Euglena gracilis* occurs at all in the dark shows that the phototransformation of straw decomposition chemicals into phytotoxic compounds, proposed by Barrett (1994) and partly supported by Everall and Lees (1997), cannot account for the inhibition observed in laboratory assays. The straw was decomposed in the dark (section 2.2) and the formation of photolytic products in the short time during the transfer into dark assay conditions is most unlikely. The current results with *E. gracilis* support those of Cooper *et al.* (1997) who showed several species of *Saprolegnia* to be inhibited by barley straw when grown in the dark.

The use of an organic growth medium in these assays meant that antibiotics produced by the microbes in the culture could potentially inhibit *E. gracilis*, especially over longer assays since dense growths of actinomycetes were observed. However, this cannot explain the inhibition after three days in the dark cultures and the apparent resistance in those cells grown in the light.

What is interesting is that after three days growth there was no inhibition of *E. gracilis* when grown in the light, but there was in the dark. After six days growth the light-grown cultures were inhibited with straw, but those grown in the dark were inhibited to a greater extent. The delayed and weaker inhibition when grown in the light suggests that light may have some protective role against litter-derived inhibitor(s). It is most unlikely that light could be transforming the inhibitory products, rendering them less potent, since this directly contradicts current opinion (Appel, 1993; Canonica *et al.*, 1995). Megharaj *et al.* (1992) showed two chlorococcalean algae to be resistant to phenolic compounds when growing photoheterotrophically but not when growing auto- or heterotrophically. Perhaps the ability to grow photoheterotrophically increases an organism's resistance to litter-derived inhibitors. Further work using axenic cultures would be valuable so more detailed measurements could be made without interference from contaminant organisms. However, the presence of decomposer organisms in the plant litter is a prerequisite for the release of inhibitor(s) so the use of axenic cultures is problematical, unless chemically solubilized lignin products could be used (e.g. Pillinger, 1993).

Overall these results show that *E. gracilis* requires a very large mass of straw to produce any inhibition and as such is ranked as only slightly susceptible to plant litter-derived inhibitors (Table 3.2 page 86).

Kingdom Eubacteria

3.6 **Division** Cyanophyta (=Cyanobacteria)

Class Cyanophyceae

In cyanobacteria the cell wall consists of four layers composed largely of lipopolysaccharides, although one layer is composed of murein which shows a similarity to gram negative bacteria (Hoek *et al.*, 1995). Members of this kingdom were tested since it appeared that cyanobacteria were particularly susceptible to decomposed plant litter (Newman & Barrett, 1993) and many bloom-forming and toxic species occur in this group.

3.6.1 **Order** Nostocales

Family Nostocaceae

Members of this family are filamentous, reproducing through the formation of hormogonia. Hormogonia are short segments of trichome (filament) formed by rounding-off of the end cells of the trichome within the surrounding mucilage sheath (Fay, 1983). Upon liberation the hormogonia develop into new trichomes. Single-celled perennating structures called akinetes can also be formed from vegetative cells which have a thick extracellular capsule and are able to withstand adverse environmental conditions. Germination of akinetes produces germlings and eventually new trichomes.

Anabaena flos-aquae

Anabaena flos-aquae is a trichome-forming (Plate 3.6), planktonic species which is prevalent in eutrophic waters, especially those enriched in organic matter (Reynolds & Walsby, 1975; Reynolds, 1984a; Paerl, 1988). Some strains of *Anabaena flos-aquae* are known to produce toxins, but the strain used in this investigation was not known to do so. It forms blooms through the presence of gas vacuoles (see section 1.2.2.1) and an unidentified planktonic *Anabaena* species had been shown to be inhibited in a reservoir where barley straw was used (Barrett *et al.*, 1996).

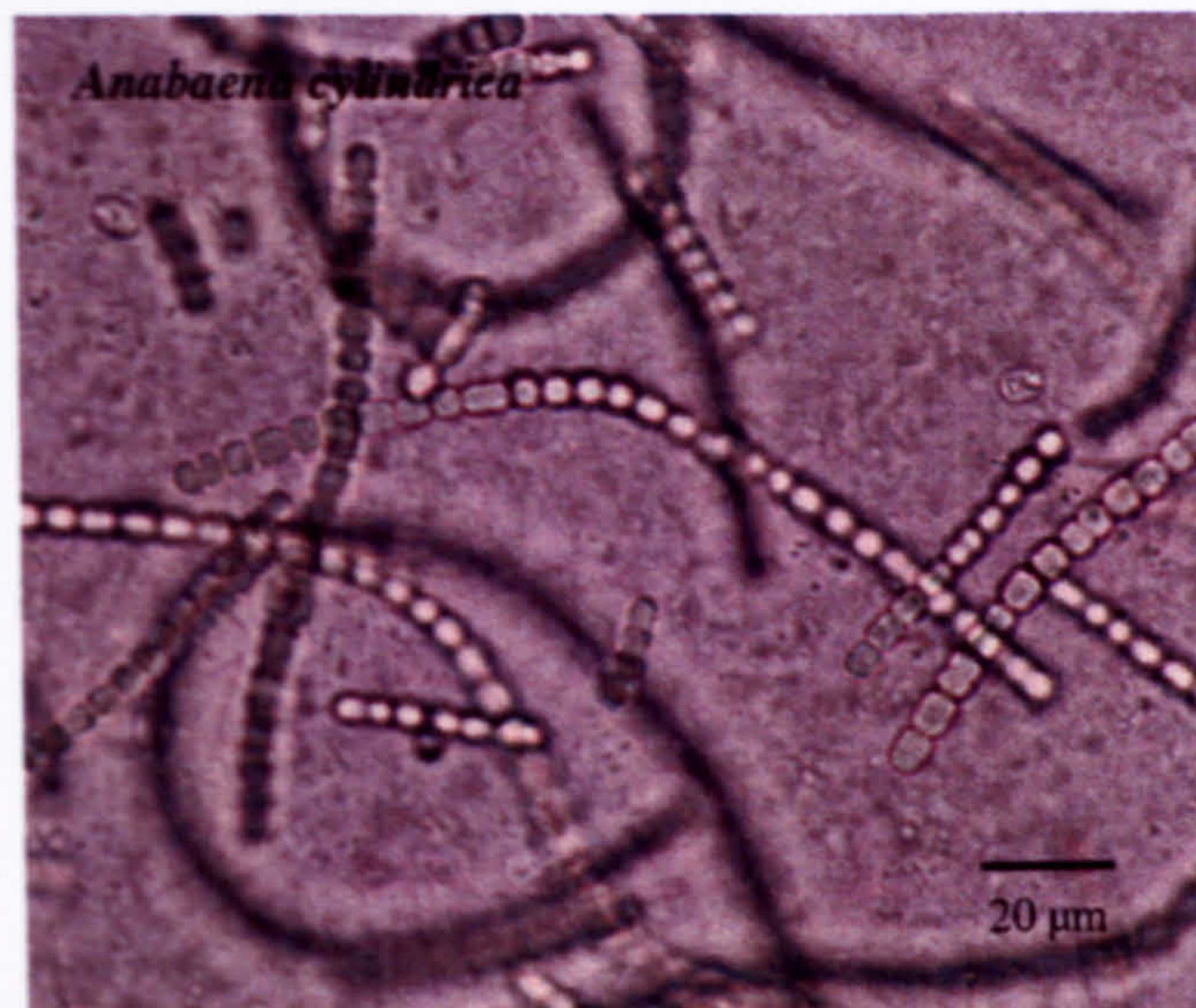
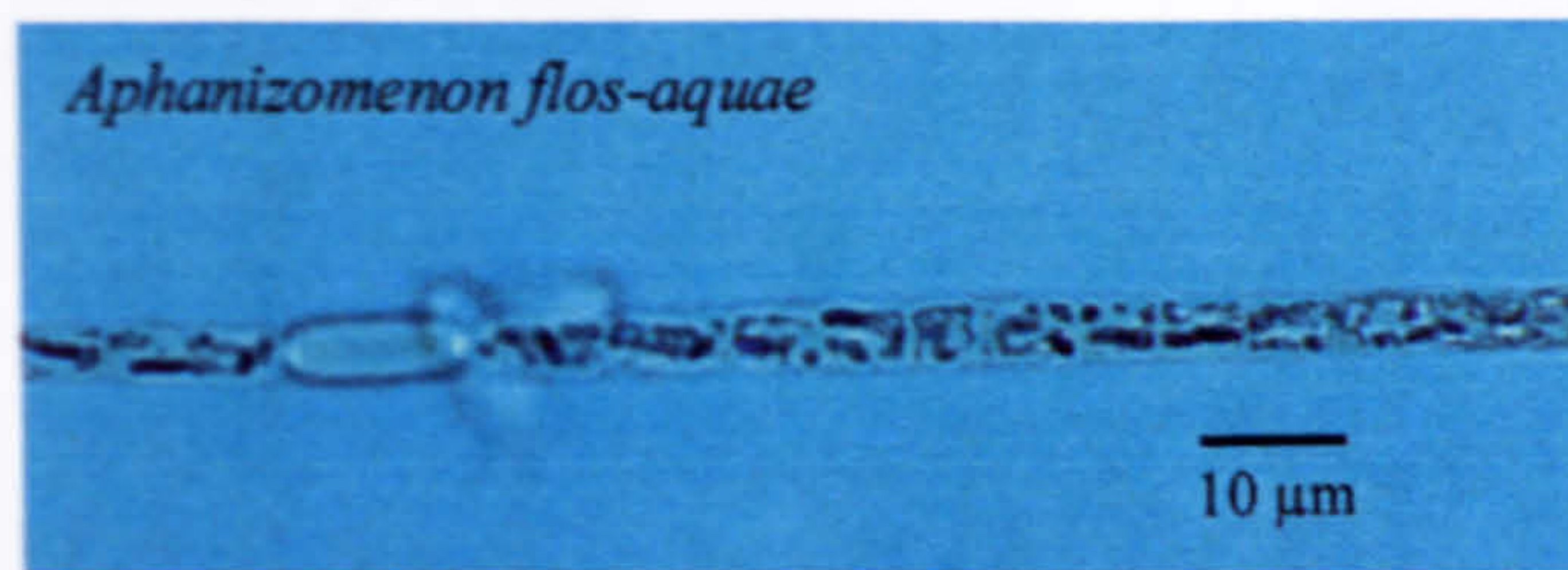


Plate 3.6 Members of the Nostocales, Cyanophyceae which were assayed.

Anabaena cylindrica

Anabaena cylindrica (Plate 3.6) can be planktonic or it can occur on aquatic surfaces such as submerged plants and stones (Geitler, 1932), although it is not known to occur in any quantity in fresh waters (Fogg, 1942). *A. cylindrica* was assayed for comparison with *Anabaena flos-aquae*, the latter belonging to the same genus but being a purely planktonic organism.

Aphanizomenon flos-aquae

A. flos-aquae (Plate 3.6) is a planktonic organism which has trichomes that aggregate into bundles or rafts. It is very common in eutrophic waters and often forms blooms (Reynolds & Walsby, 1975; Fay, 1983; Paerl, 1988).

3.6.2 Results for Nostocales

Both the planktonic members of the Nostocaceae, *Anabaena flos-aquae* and *Aphanizomenon flos-aquae* were shown to be inhibited (Figure 3.17), with *Anabaena flos-aquae* appearing to be the most susceptible to barley straw (Table 3.2 page 86). The results for *Anabaena cylindrica* are the opposite of those for *Anabaena flos-aquae* since growth stimulation was observed (Figure 3.17). *A. cylindrica* showed a stimulation in growth of nearly 400% in the presence of 1800 g dry mass m⁻³ barley straw, a concentration which inhibits over half the other algae tested (Table 3.2 page 86).

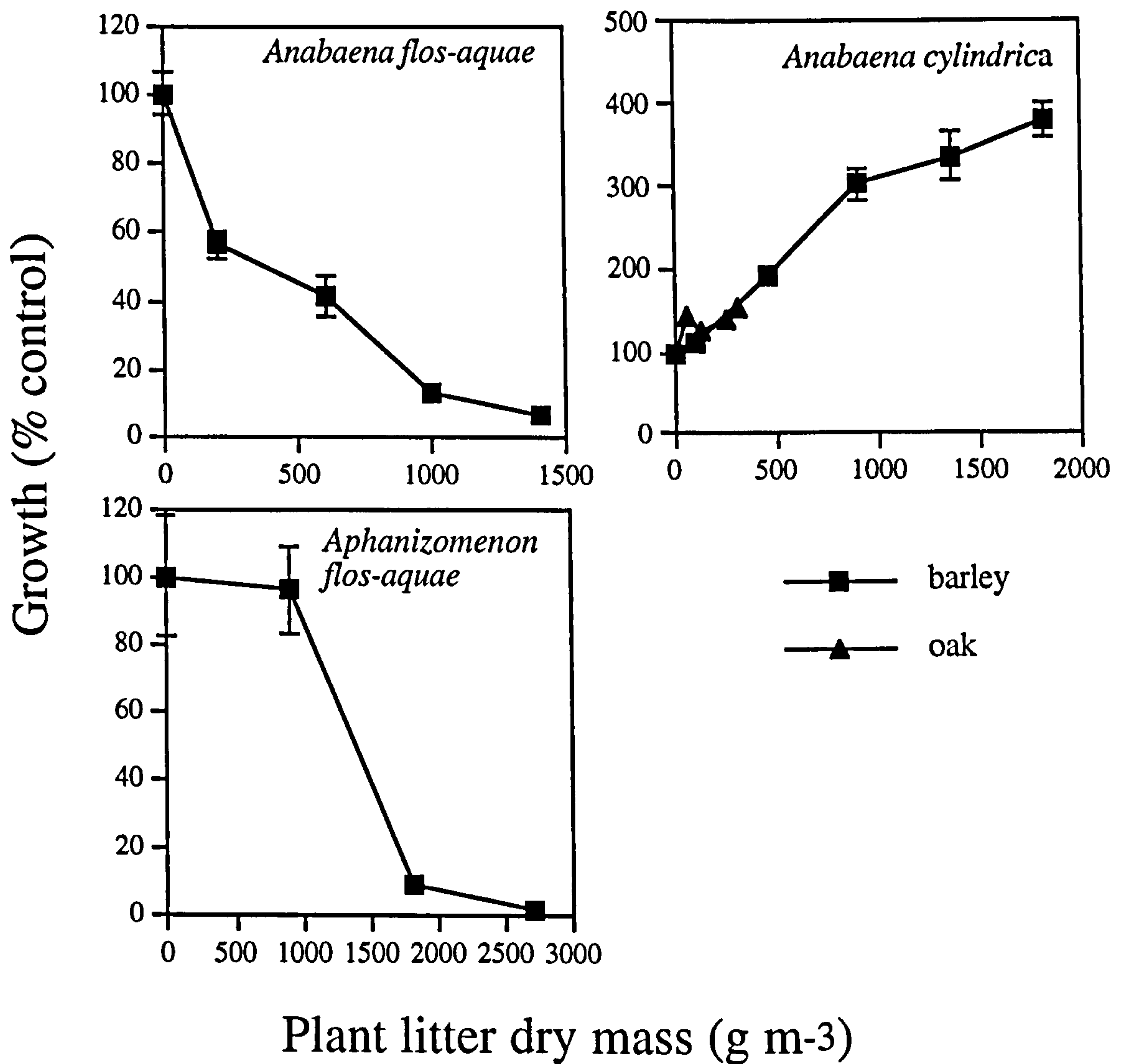


Figure 3.17 Growth of *Anabaena flos-aquae*, *Anabaena cylindrica* and *Aphanizomenon flos-aquae* with different masses of barley straw and oak leaf FPOM. Values shown are means ($n=5$) \pm SE. All graphs show a significant difference from the control values ($p < 0.001$ or $p < 0.01$ for *A. cylindrica* with oak leaf FPOM).

3.6.3 Order Oscillatoriales

Family Oscillatoriaceae

Reproduction is by the formation of hormogonia and akinetes are not produced by members of this order (Fay, 1983; Hoek *et al.*, 1995). *Oscillatoria* spp. may vary both morphologically and in their ecological responses depending on the strain of a species used and the conditions under which it is grown (see Whitton & Peat, 1969; Gibson & Smith, 1982).

Oscillatoria redekei

O. redekei (Plate 3.7) is a planktonic organism which favours highly eutrophic waters (Whitton & Peat, 1969). It often becomes dominant in shallow, turbid water bodies (Gibson & Smith, 1982; Scheffer *et al.*, 1997) where its high adaptive ability to survive under low levels of insolation gives it a competitive advantage (Reynolds, 1994b).

Oscillatoria animalis

Oscillatoria animalis (Plate 3.7) is a freshwater species but is occasionally found in brackish water (Drouet, 1968). It is largely a surface-associated species in freshwaters, although it may occur sub-aerially and can survive a wide range of temperature, often being found in hot sulphurous springs (Geitler, 1932). This species was tested as a comparison with *O. redekei* which is a wholly planktonic species.

3.6.4 Results for Oscillatoriales

Oscillatoria animalis was not susceptible to decomposed barley straw since growth increased nearly six-fold in its presence (Figure 3.18). This is the opposite to *O. redekei* where 50% growth inhibition was obtained with as little as 670 g dry mass m⁻³ of barley straw (see Table 3.2 page 86).

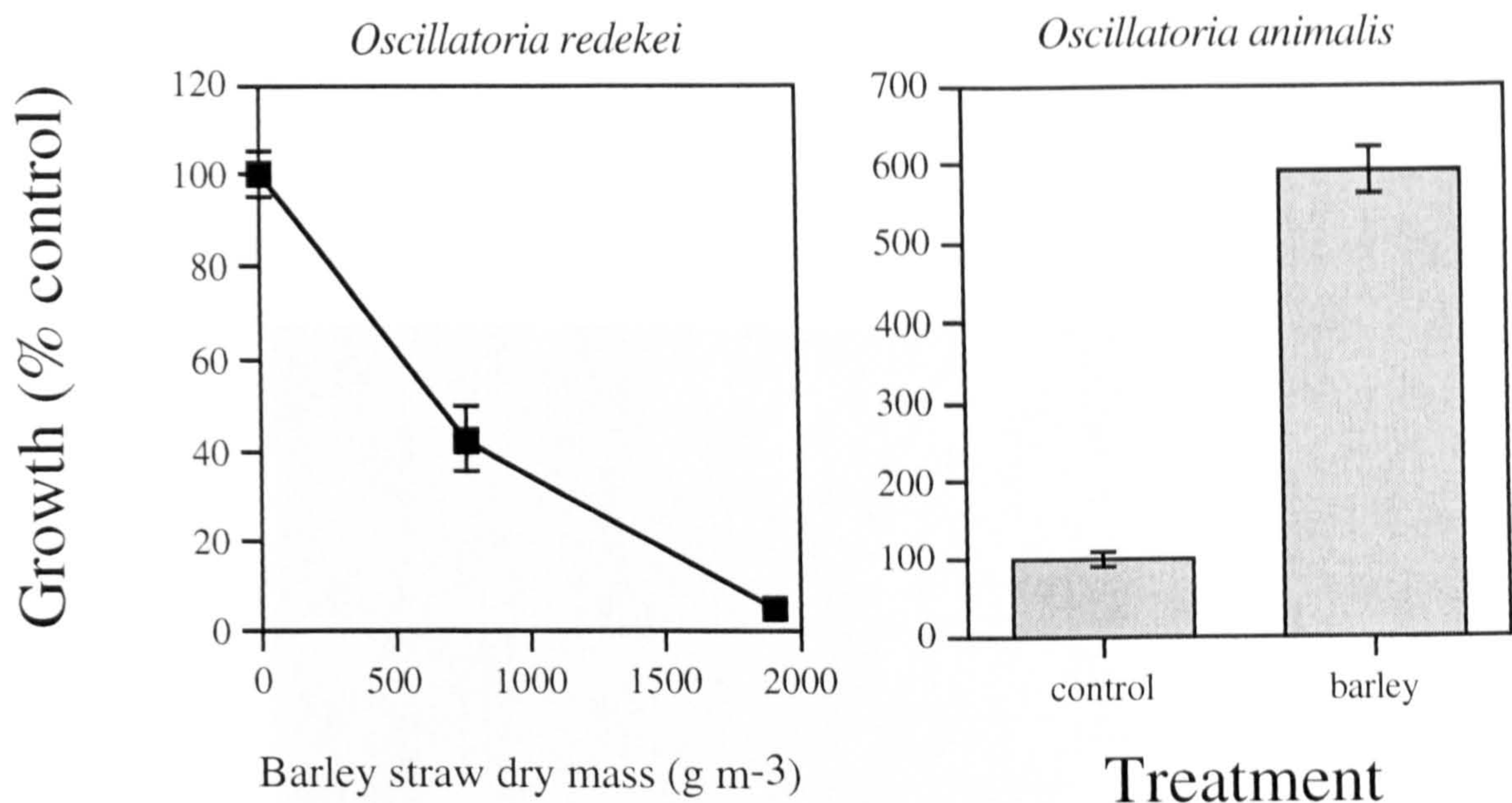


Figure 3.18 Growth of *Oscillatoria redekei* and *Oscillatoria animalis* with barley straw. *O. animalis* grown with 2727 g dry mass barley straw m⁻³. Values are means \pm SE (n=5). Inhibition of *O. redekei* is significant ($p < 0.001$) and stimulation of *O. animalis* is significant ($p < 0.001$).

3.6.5 Order Chroococcales

Family Chroococcaceae

Synechococcus sp.

Synechococcus spp. are unicellular species, though sometimes the cells occur in pairs (Plate 3.8) and reproduction takes place through binary fission (Fay, 1983). The particular strain used in this investigation was originally a 'weed' which grew in a contaminated culture of *Microcystis aeruginosa* after accidental heating to ca. 35°C for three days. The two most common species in Britain are *S. aeruginosa* and *S. elongatus* (Pentecost, 1984), both of which occur frequently in the plankton and on damp surfaces such as rocks and soil. *Synechococcus* is a known bloom-forming genus and was used as a comparison with *Microcystis aeruginosa* which is a planktonic species belonging to the same family (see below).

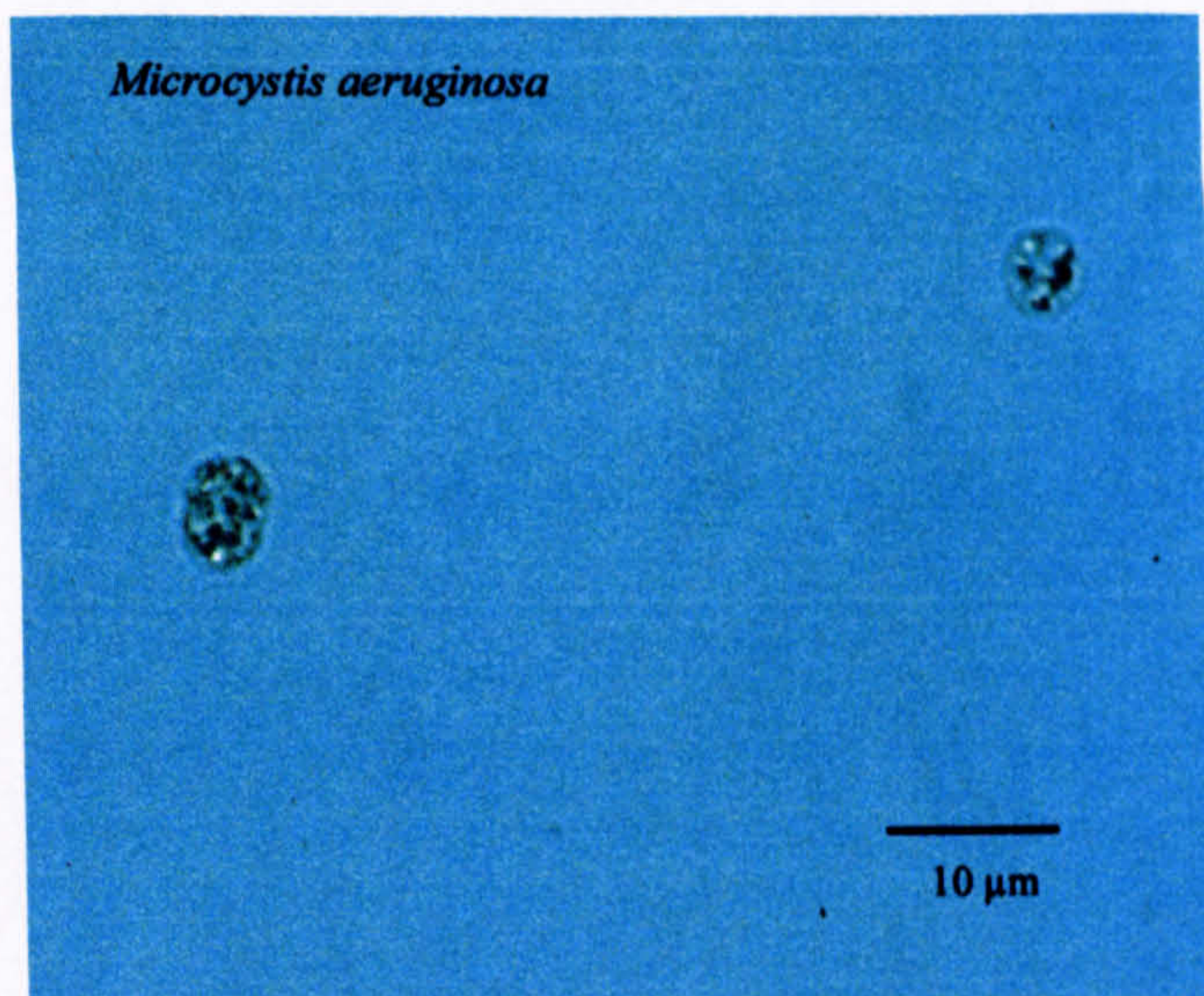
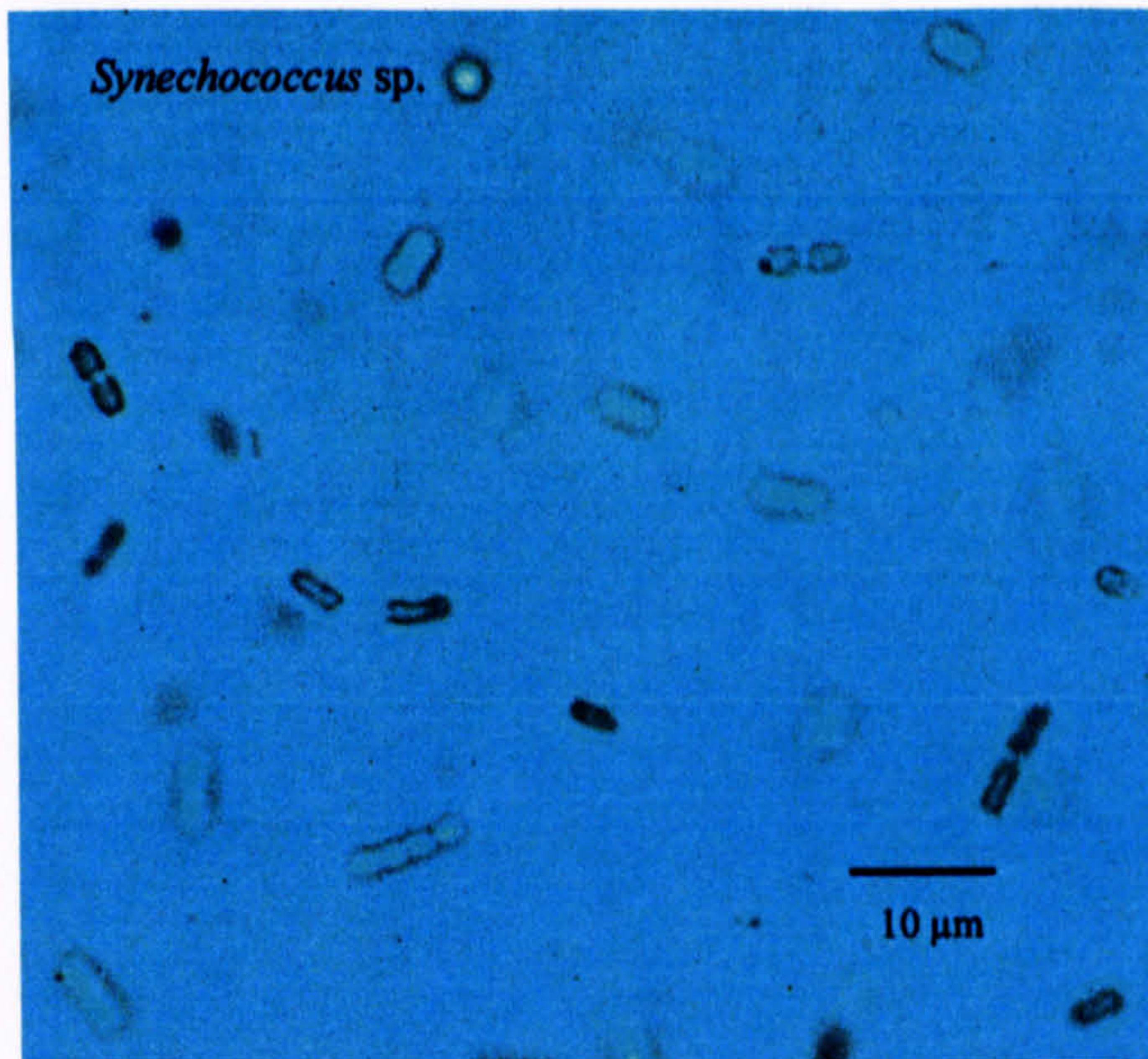


Plate 3.8 Members of the Chroococcales, Cyanophyceae which were assayed.

Microcystis aeruginosa

In the wild *Microcystis aeruginosa* cells are held within a common mucilage mass to produce colonies of varying size and structure (Reynolds *et al.*, 1981; Prescott, 1969). When put into culture most strains of *M. aeruginosa* tend to lose the mucilaginous mass which surrounds them and they grow separately as single cells (Plate 3.8) (Walsh & Merrill, 1984). *M. aeruginosa* overwinters on sediments (Reynolds *et al.*, 1981), and is common in the plankton of eutrophic lakes and slow-flowing waters (Fay, 1983; Reynolds, 1984a; Paerl, 1988). It often increases towards the end of summer, forming blooms which can be toxic, therefore, the control of growth in potable water supplies is of the utmost importance. *M. aeruginosa* had previously been shown to be inhibited with barley straw (Newman & Barrett, 1993), however, the opportunity arose to test several different strains of the same species, thus, information could be collected on intraspecies susceptibility.

Strain *Loe pool*

This strain was collected from a bloom which occurred during August 1996 from Loe pool, a freshwater lake in Cornwall, U.K. (lat. 50° 4' N, long. 5° 17' W; see Florey & Hawley, 1994 and John *et al.*, 1998 for site details). Microscopical examination showed the sample to be virtually unialgal. Tests carried out by the Environment Agency had shown *M. aeruginosa* from Loe Pool to be able to produce toxins (T. Rennals, pers. comm.) but it was not known whether this particular sample was able to do so. This strain was assayed (Chlorophyll *a* extraction) with straw after only one sub-culture in the laboratory, while it still retained its 'floc' forming habit (i.e. still grew in a mucilaginous mass). By testing *M. aeruginosa* while it still retained its colonial habit the hypothesis that mucilage may afford some protection to the cyanobacterium could be tested.

Strain *AK1*

This strain of *M. aeruginosa* was tested since it was a known toxin-producing strain. It was not known whether the production of toxins would change the sensitivity of *M. aeruginosa* toward the litter-derived inhibitors since, to my knowledge, no toxic strains had ever been assayed. This particular strain of *M. aeruginosa* produced the hepatotoxin microcystin in

culture at approximately 0.2 µg microcystin-LR equivalents mg⁻¹ dry mass (G. A. Codd, pers. comm.).

Strain *CCAP 1450/6*

This strain was obtained from CCAP (see Table 2.5) and was the original strain used in the study by Newman & Barrett (1993). It is known to be inhibited by barley straw and as such was tested again for comparison with the present bioassays, since those of Newman & Barrett (1993) were performed slightly differently.

Strain *Sciento*

This strain was tested as a comparison with the other strains and was not known to produce toxins

3.6.6 Results for Chroococcales

Both *Microcystis aeruginosa* and *Synechococcus* sp. were inhibited by barley straw (Figure 3.19). Both species are classed as very susceptible but *M. aeruginosa* is the most susceptible of all the 'algae' tested (see Table 3.2 page 86). All of the strains of *M. aeruginosa* were inhibited by roughly the same amount apart from the Loe pool strain, which appeared to be less sensitive to higher doses of straw (see Figure 3.19).

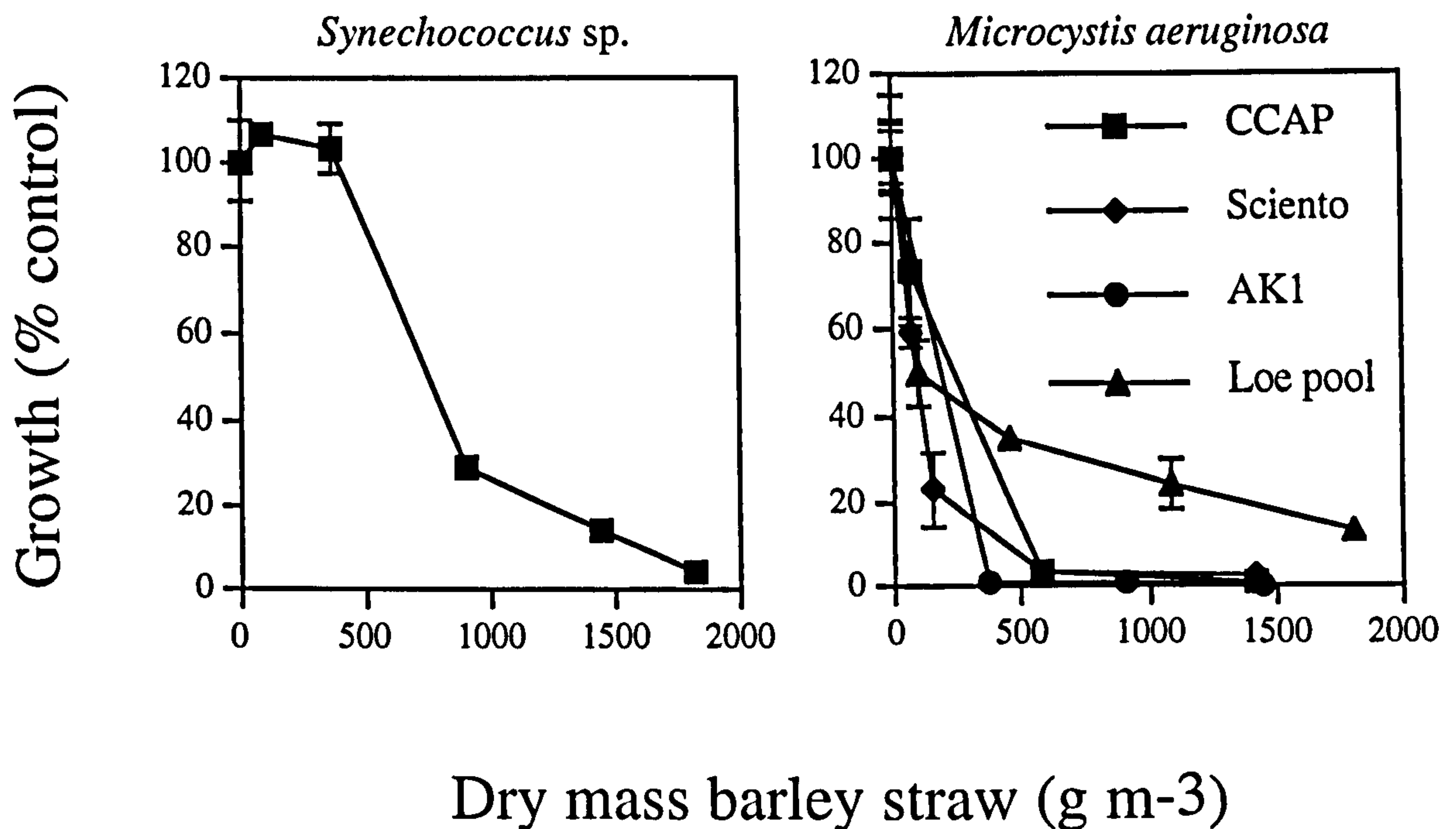


Figure 3.19 Growth of *Synechococcus* sp. and four strains of *Microcystis aeruginosa* with different masses of barley straw. Values shown are means \pm SE (n=5), inhibition is significant in all cases ($p < 0.001$).

3.6.7 Discussion for the Cyanophyceae

As a group the Cyanophyta show highly variable responses to plant litter-derived inhibitors, with some species being very susceptible (e.g. *M. aeruginosa*, *O. redekei*), whereas others are stimulated in its presence (e.g. *A. cylindrica*, *O. animalis*).

Ecologically *Aphanizomenon flos-aquae* generally requires the same growth conditions as *Anabaena flos-aquae* with the two species often co-occurring (Reynolds & Walsby, 1975; Paerl, 1988). Although their ecology may be similar *Aphanizomenon flos-aquae* requires over three times more barley straw to achieve the same degree of inhibition that is seen in *Anabaena flos-aquae* (see Table 3.2 page 86). For these two species at least the similarity of ecology does not appear to be linked to the relative sensitivity to litter-derived inhibitors.

The different strains of *Microcystis aeruginosa* that were tested appear to have roughly the same susceptibility. Any variation can probably be ascribed to the variation

which occurs naturally in the barley straw (see section 3.7.1). For the Loe Pool strain the apparent inability to produce near total inhibition (Figure 3.19) is probably a side effect of the way the assay was carried out. The Loe pool strain was still in its floc-forming habit and therefore cell counting was not possible so chlorophyll *a* measurements had to be used. Discrepancies could therefore exist between this strain and the other strains, where biomass was estimated using cell counts (see section 2.3.3). However, what is more likely is that near-total inhibition was not obtained due to chlorophyll *a* contamination from other more resistant species of algae. Although the culture was from a bloom of *M. aeruginosa*, no bloom in the field will be totally unialgal. The presence of small amounts of resistant contaminant species, growing over the time-course of an assay, may have produced the amounts of chlorophyll *a* detected with higher masses of straw.

The susceptibility of the AK1 strain (and possibly the Loe Pool strain, see section 3.6.5) to barley straw shows that the inhibitor(s) do work against microcystin-producing strains. To my knowledge this is the first time plant litter-derived inhibitor(s) have been shown to work against a toxin-producing cyanobacterium. Whether the inhibitor(s) are active against species which produce other toxins (e.g. *Anabaena* spp., *Aphanizomenon* spp.) or greater amounts of toxins is not known and requires further study.

3.7 Discussion for all taxa

3.7.1 Variability of the assays

One inherent problem with all of these assays is the use of live material as a source of toxicant. Both barley straw and oak leaves require microbial activity to be present for the release of the inhibitory material (Gibson *et al.*, 1990; Pillinger *et al.*, 1992). Since the plant litter was decomposed under septic conditions, the microflora developed naturally and would change over time and within and between assays. This inevitably introduces variation into the assays, although the algae were sampled at specific stages in their growth

cycle to minimize variation and straw was used only where it had already been shown to be inhibitory.

3.7.1.1 50% inhibition

The algae were ranked and categorized using 50% inhibition with straw, since not all of the species were tested with oak leaf FPOM. Algae were placed into four categories for comparison since the variation in the plant litter meant the 50% inhibition values were prone to error. The calculation of 50% inhibition values was also prone to error since they were calculated from graphs of raw data. The data were not fitted with curvilinear regression since the curves were variable in shape. Attributing a polynomial function to the data would introduce more variation when calculating just one point i.e. 50% inhibition, as opposed to comparisons between entire curves. Furthermore, some species had been tested with only one or two different masses of plant litter to determine whether they were resistant or not, making regression inappropriate. Putting the algae into different categories (see Table 3.2 page 86) allowed general comparisons to be made without requiring precise 50% inhibition values.

3.7.2 Oak leaf versus barley straw

Not all of the species were tested with oak leaf FPOM since it was found that both oak leaf and barley straw appeared to produce the same effects on each individual species, whether it was growth promotion or inhibition. The species ranked in order of susceptibility with barley straw showed broadly the same rankings with oak leaf FPOM. The exceptions to this appear to be *Asterionella formosa* which required a relatively higher amount of Oak leaf FPOM and *Stichococcus bacillaris* which required a relatively lower amount of oak leaf FPOM when compared to barley straw and the other species (see Table 3.2 page 86). In hindsight it would have been better to assay all of the species with oak leaf FPOM in order to achieve a more accurate ranking of the species.

Overall, to achieve similar levels of inhibition or growth promotion a smaller dry mass of oak leaf FPOM was required than for barley straw. The methodology in this study meant that the liquor in which oak leaves were rotted was used as the experimental medium after the addition of algal nutrients (see section 2.3.2.2). It is likely that soluble inhibitor(s) already present in the liquor at the start of the assay could add to the effect of the particulate matter (Newman & Barrett, 1993). Ridge *et al.* (1995) found that liquor with all the particulate matter filtered out was sometimes, but not consistently, inhibitory to *Chlorella pyrenoidosa*, depending on the stage of leaf decomposition. It is conceivable that the inhibitor(s) released from decaying oak leaves are chemically different to those from barley straw. However, although the inhibitors may differ in detailed chemical structure, the oxidized polyphenolic mode of action is thought to be based on the same principle in both straw and leaf litter (Pillinger *et al.*, 1994; Ridge *et al.*, 1995) and in brown-rotted wood (Ridge & Pillinger, 1996). It may be that the FPOM from oak leaves has a higher proportion of readily solubilized polyphenolic compounds (Webster & Benfield, 1986) from which inhibitor(s) can be released than does barley straw; or the phenolics may be more readily oxidized to produce a greater stimulatory/inhibitory effect.

3.7.3 Growth stimulation with low doses of plant litter

A number of the susceptible species tested showed a stimulation in growth with small amounts of barley straw (*Asterionella formosa*, *Stichococcus bacillaris*, *Synechococcus* sp.) and oak leaf FPOM (*Chlorella vulgaris*). This same stimulation in growth with small amounts of barley straw was observed by Pillinger (1993). Schulz (1888, cited in Wiedman & Appleby, 1972) proposed that all poisons are stimulatory in small quantities and this effect is now well-established being called a hormetic or oligodynamic effect (Wainwright, 1994). Various herbicides, insecticides, fungicides and antibiotics have been shown to be stimulatory to plants at sub-inhibitory concentrations (Wiedman & Appleby, 1972 and references therein) so this effect is likely to be applicable to the plant litter assays shown in this chapter. The effect is not shown in all of the species tested, possibly because the amounts of plant litter that were used were not small enough or the hormetic effect is not seen in all species.

3.7.4 Resistance

All of the resistant species, where biomass estimation was carried out, showed a stimulation in growth in the presence of both oak leaf FPOM and barley straw (*Scenedesmus quadricauda* is classed as resistant but see section 3.3.3 and Figures 3.10 & 3.11 since the results were variable). The observed stimulation could be due to the fact that these species were more resistant to the inhibitors and stimulation could be attributed to a hormetic effect, with susceptibility becoming apparent at higher concentrations of the inhibitor(s). However, this seems most unlikely since very large amounts of plant litter (over 2000 g m⁻³) produced a stimulation in growth and, even if the hormetic effect were real, these species would still be only slightly susceptible.

It is unlikely that the introduction of plant litter would contribute stimulatory amounts of major inorganic nutrients into the growth medium. Growth stimulation could be due to the presence of some unknown organic nutrient or perhaps certain organic substances originating from the plant litter are acting in a growth regulatory capacity (Larson, 1978).

3.7.4.1 Assimilation of organic molecules

The increase in biomass of resistant species could be due to the algae being stimulated by the inhibitor(s) or by other associated organic matter. Of the resistant species *Nitzschia filiformis* (Lewin & Lewin, 1960), *Scenedesmus quadricauda* (Droop, 1974; Pollinger & Berman, 1976; Soeder & Hegewald, 1988) and *Anabaena cylindrica* (Fogg, 1944) have all been shown to be able to assimilate organic matter and grow heterotrophically and/or photoheterotrophically (the status of *Oscillatoria animalis* is unknown). As well as the resistant species, the slightly susceptible species *Euglena gracilis* (Droop, 1974; Hoek *et al.*, 1995) and *Chlorella vulgaris* (Droop, 1974; Mayo & Noike, 1994) have also been shown to be able to assimilate organic matter. Resistance may, therefore, be related to the ability of an alga to assimilate organic matter. However, *Pediastrum boryanum* (Pollinger & Berman, 1976), *Aphanizomenon flos-aquae* (Smith, 1982), *Anabaena flos-aquae*

(Droop, 1974; Smith, 1982) and *Synechococcus* sp. (Droop, 1974) have all been shown to be able to assimilate organic matter, but they are all susceptible to plant litter-derived inhibitors. Not all algae can assimilate the same organic molecules or utilize them in meaningful amounts (Droop, 1974). For instance, *Aphanizomenon flos-aquae* and *Anabaena flos-aquae* can assimilate glucose but probably not at ecologically important rates (Smith, 1982). The degree of resistance could be related to the ability of an alga to assimilate the organic molecules present in decomposed plant litter as an energy source and/or as a source of carbon (in addition to or instead of carbon dioxide). Further investigations using axenic cultures would have to be carried out to test for the uptake and incorporation of organic molecules from plant litter.

3.7.4.2 Detoxification of inhibitor(s)

Another possible mechanism to overcome resistance is detoxification of the inhibitor(s). Some species of algae are known to be able to detoxify organic molecules which are inhibitory to other algae. Previous workers have shown that a range of phenolic materials can be degraded by *Scenedesmus* sp. and *Chlorella vulgaris* (Klekner & Kosaric, 1992a, 1992b), especially when growing photoheterotrophically, as is the case for *C. vulgaris* (Megharaj *et al.*, 1992). *Anabaena cylindrica* and *Euglena gracilis* have been shown to degrade phenolic compounds, though this ability may be diminished in the continued presence of the compound (Ellis, 1977), which may help to explain the greater inhibition of *E. gracilis* when grown in the light after six days (see Figure 3.16). The recovery in cell numbers seen in *Scenedesmus quadricauda* (e.g. Figure 3.10) could, perhaps, be explained by the induction of detoxifying processes. Since all of the species mentioned above are either resistant or only slightly susceptible, the possibility exists that toxicant degradation is one of the methods of overcoming the inhibitor(s). Until the chemical nature of the inhibitor(s) is defined more precisely, or the breakdown products in the presence of different species of algae are assayed, this remains only a possibility.

3.7.5 Comparisons between species

Table 3.2 (page 86) presents all of the species tested, categorized according to their relative sensitivities to decomposed plant litter. The question is whether this can answer the first aim of this thesis (section 1.5) which was to identify any similarities in those species which are susceptible and those which are resistant.

3.7.5.1 Interspecies comparisons

It appears from the results presented in this chapter and by Gibson *et al.* (1990) that sensitivity to plant litter-derived inhibitors is not related to general taxonomic characteristics. The initial idea that diatoms appear to be resistant has proven to be incorrect since of the three species of diatoms tested, two were found to be susceptible (*Asterionella formosa* and *Tabellaria flocculosa*) and one was found to be resistant (*Nitzschia filiformis* var. *conferta*) (section 3.1.2).

Opposing results have been obtained with members of the same genus. The cyanobacterium *Oscillatoria redekei* was found to be very susceptible whereas growth of *Oscillatoria animalis* was stimulated in the presence of barley straw. The same effect has been shown in the genus *Anabaena*, where *Anabaena flos-aquae* was found to be very susceptible but growth of *Anabaena cylindrica* was stimulated (see Chapter 4 for further investigations). The idea that cyanobacteria are particularly susceptible to litter-derived inhibitors (Newman & Barrett, 1993) may be an artefact of the species tested, since the current results show some cyanobacteria to be resistant. The two different species of *Scenedesmus* also show differences, but less dramatically than those mentioned above. The results for *S. quadricauda* are variable, as mentioned in section 3.3.2, whereas *S. subspicatus* always showed a stimulation of growth in the presence of plant litter.

The only tentative correlation that can be drawn from this work is that the chlorococcalean algae appear to be less sensitive than other groups of algae. However, there are resistant algae from other taxa so perhaps this is just an artefact of the number of

Category	Species/strain	Plant litter dry mass required for 50% inhibition of growth (g m ⁻³ medium)	
		barley straw	oak leaf
Very susceptible 0-1000	<i>Microcystis aeruginosa</i> (P)		
	Sciento	70	-
	Loe pool	90	-
	AK1	180	-
	CCAP 1450/6	230	-
	<i>Tabellaria flocculosa</i> (P)	295	18
	<i>Anabaena flos-aquae</i> (P)	375	-
	<i>Closterium ehrenbergii</i> (T&P)	450	-
	<i>Cosmarium biretrum</i> (T)	540	50
	<i>Asterionella formosa</i> (P)	667	90
	<i>Oscillatoria redekei</i> (P)	670	-
	<i>Spirotaenia erythrocephala</i> (T)	750	-
	<i>Synechococcus</i> sp. (P&S)	750	-
	<i>Staurastrum pingue</i> (P)	960	60
Susceptible 1001-2000	<i>Aphanizomenon flos-aquae</i> (P)	1370	-
	<i>Pediastrum boryanum</i> (P)	1500	130
	<i>Stichococcus bacillaris</i> (S)	1940	80
Slightly susceptible >2001	<i>Euglena gracilis</i> ¹ (P&S)	>2700 ¹	-
	<i>Chlorella vulgaris</i> (P&S)	>5400	400
Resistant	<i>Nitzschia filiformis</i> var. <i>conferta</i> (S)	-	-
	<i>Scenedesmus subspicatus</i> (P)	-	-
	<i>Scenedesmus quadricauda</i> ² (P&S)		
	CCAP 276/21	-	-
	Sciento	-	-
	<i>Hydrodictyon reticulatum</i> ³ (P)	-	-
	<i>Anabaena cylindrica</i> (P&S)	-	-
	<i>Oscillatoria animalis</i> (S)	-	-

Table 3.2 Susceptibilities of algal and cyanobacterial species to barley straw and oak leaf FPOM. Species are separated into categories according to the amount of barley straw required to obtain 50% inhibition of growth (see section 3.7.1.1). Letters in parentheses show the 'ecology' of each species, P=planktonic, S=surface associated, T=occurs in tychoplankton. ¹ result obtained for *Euglena gracilis* grown in the light; ² results for *Scenedesmus quadricauda* were variable (see Figures 3.10 & 3.11 and section 3.3.2); ³ results for *Hydrodictyon reticulatum* were obtained differently to the other species (see section 3.3.2).

chlorococcalean species tested. Further work testing a wider range of species is required before any firm conclusions can be made.

3.7.5.2 Intraspecies comparisons

All four strains of *Microcystis aeruginosa* were found to be very susceptible, suggesting that for this species there are no dramatic differences in susceptibility within a species. Slight differences are shown in Table 3.2 but these can be ascribed to the variation present in the plant litter and/or the method of assaying growth. Both strains of *Scenedesmus quadricauda* showed no differences in their growth with plant litter, though the results were variable. The *Chlorella vulgaris* strain used in this thesis (and in Pillinger (1993)) also appears to show no difference in susceptibility when compared with the strain used by Gibson *et al.* (1990), since both appear to be only slightly susceptible to barley straw. Exact 50% inhibition values cannot be calculated from the data, but from Table 3.2 it can be seen that over 5400 g m⁻³ of barley straw is required to obtain a 50% inhibition in growth of *C. vulgaris*, whereas over 4000 g m⁻³ of barley straw was required to obtain the same degree of inhibition in the strain used by Gibson and co-workers.

The current results and comparisons with other workers suggest that there are no consistent intraspecies differences in algal susceptibility to litter-derived inhibitor(s). However, the strains tested here are only a tiny fraction of the different strains of algae which are known to exist. Much more work will have to be carried out with strains of algae from a wide range of habitats to gain a better understanding of intraspecies variation.

3.7.5.3 Ecology

Those algae common in eutrophic habitats and with the ability to grow to nuisance proportions fall within all the categories of susceptibility or resistance (Table 3.2). One of the only common features of the resistant species appears to be their association with surfaces. *Nitzschia filiformis* var. *conferta* (Kramer & Lange-Bertalot, 1988) and *Oscillatoria animalis* (Geitler, 1932) are purely surface associated species whereas, of the

other resistant species, *Anabaena cylindrica* (Geitler, 1932) and *Scenedesmus quadricauda* (Philipose, 1967) are known to occur both planktonically and on surfaces. Organic matter will sediment out onto surfaces and, in freshwater ecosystems, most decomposition is carried out by bacteria associated with surfaces (Wetzel, 1992); consequently, surface-associated algae may have evolved mechanisms to overcome any inhibitory decomposition products that may be released. However, this cannot explain resistance in all cases since both *Scenedesmus subspicatus* and *Hydrodictyon reticulatum* are resistant but they are planktonic species.

3.7.6 Extrapolation into the field

The actual amounts of material needed for algal inhibition in these laboratory studies are larger than those which are reported to be necessary in the field (Ridge *et al.*, 1995; Barrett *et al.*, 1996; Everall & Lees, 1996, 1997). In the field, straw is applied to the surface waters since aerobic conditions are necessary for its inhibitory action; thus, the inhibitory component is more likely to concentrate in the surface layers, producing a relatively higher concentration of inhibitor than that calculated for the entire waterbody. Algae generally grow in, or at some point pass through, the surface layers and are therefore likely to come into contact with this area of high inhibitory activity. Any concentrating effect will depend on the physical conditions of the water body, such as stratification, the amount of turbulence and the depth of the water, so this would not hold true in all cases.

Geyer *et al.* (1985) suggested from work by Jouany *et al.* (1983) that organic chemicals would be more toxic under the dynamic conditions that occur in the field; a static test would underestimate toxicity towards the algae. In the field more complex situations are encountered, where several toxicants may be present simultaneously and where interactions in processes such as degradation, concentration and detoxification may occur. Additionally, variations in temperature, illumination and nutrient levels may affect susceptibility to a toxicant. Whether susceptibility to the organic chemicals released from plant litter is increased under field (dynamic) conditions is not known and requires further investigation.

It is also probable that algae respond differently to toxins when grown in unialgal culture rather than in multi-species tests. Walsh & Merrill (1984) suggested that single species in culture do not respond to the physical and chemical conditions of the environment with the same sensitivity as they would if in competition with other species (i.e. in the field).

The difference in dosage highlights the problems of extrapolating these laboratory investigations into a field situation. Further evidence is required to see if the relative sensitivities presented in this thesis correlate with those species found in the field where barley straw has been used. However, of the species found to be susceptible in this thesis (Table 3.2) *Asterionella formosa*, *Tabellaria* sp. (Barrett *et al.*, 1996) and *Aphanizomenon flos-aquae* (Everall & Lees, 1996) have all been shown to be susceptible in reservoirs treated with barley straw, thus, reinforcing the current laboratory investigations.

Chapter 4

The effects of litter-derived inhibitor(s) on cell and colony structure

4.1 Introduction

Although the inhibitory/stimulatory effects of plant litter are clearly shown by the previous results (Chapter 3) they give few clues as to their mode of action. Changes at the cellular level in inhibited algae have not been studied before since only the effects on biomass were considered important to people using plant litter in the field. The current work where many species were tested provided an ideal opportunity to look for any such cellular changes. Most of the species tested were quantified by counting cells, which meant that a large number of cells could be quickly examined and any gross changes in cell anatomy/structure could be observed. Any observed changes were investigated further and the results are described in this chapter. For most of the test species there were no detectable changes in morphology and/or size of the cells using light microscopy. No further investigations were deemed necessary if no visible changes were detected, although ultrastructural investigations were carried out on some species (see section 4.4).

When assays were carried out with plant litter it was not known whether the observed cellular changes could be attributed specifically to the plant litter-derived inhibitor(s) or whether they were a general effect caused by overall growth inhibition. As a means of comparison, the test species' were grown with copper sulphate, which has often been used in the past to control nuisance growths of algae (Boney, 1989). Excess copper is known to inhibit a large number of enzymes and disrupts several aspects of plant biochemistry including photosynthetic electron transport, pigment synthesis and membrane integrity via peroxidative damage by free radicals (McBrien & Hassall, 1965; Woolhouse, 1983; Fernandes & Henriques, 1991).

4.2 Changes in cell size/morphology

A number of species appeared to show changes in their cell and/or colony size and morphology. For those species which were investigated further the results are shown below.

4.2.1 *Stauroastrum pingue*

Plate 4.1 (control) shows a typical cell of the desmid *Stauroastrum pingue* exhibiting three processes from each semicell (one process in each semicell is 'pointing out of the page' and is therefore not focused and cannot be seen-cf. Plate 3.2). When *S. pingue* was grown in the presence of inhibitory amounts of plant litter (section 3.2) or copper sulphate the processes showed a definite change in size and morphology (Plate 4.1).

To measure the length of the cell processes the distance between the tips of two processes in each semicell (see Plate 4.1(control)) was measured randomly for a number of cells across a microscope slide. The measurement between the processes did not give an absolute measure of their length since a change in angle could affect this; however, from observing a number of cells it was apparent that this was a fast and efficient method to detect changes in process length, since the changes were usually large and quite obvious. The main body of the whole cell was also measured as is shown in Plate 4.1(control).

Results

It can be clearly seen that when *S. pingue* was inhibited by plant litter (growth was 40% and 25% of the control value with oak FPOM and barley straw respectively) the growth of the processes was significantly reduced (Figure 4.1). The reduction in the length of the main body of the cell is small but it is significant. From figure 4.1 (c) it can also be seen that copper sulphate gave the same effect, producing a decrease in cell dimensions (overall

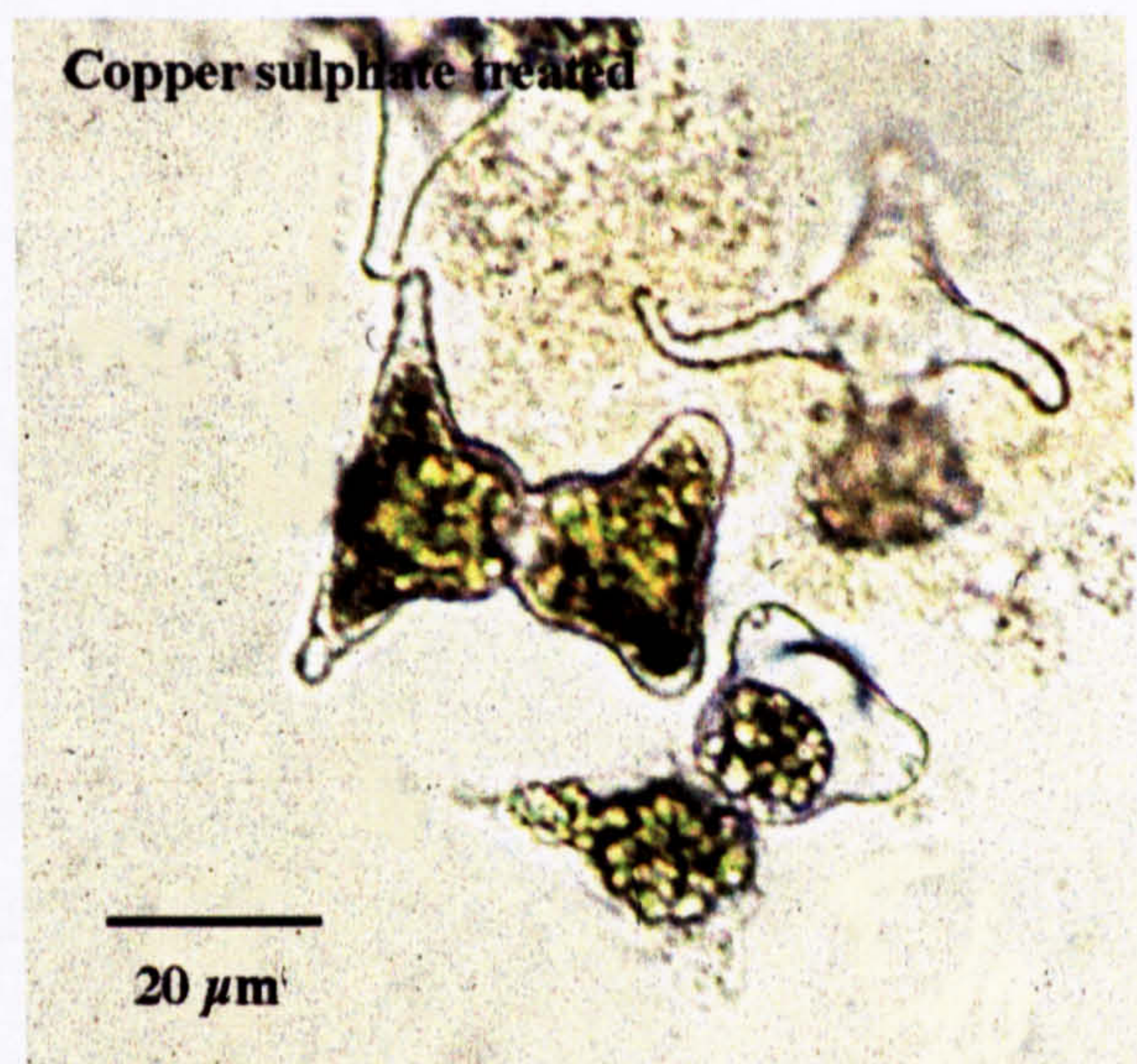
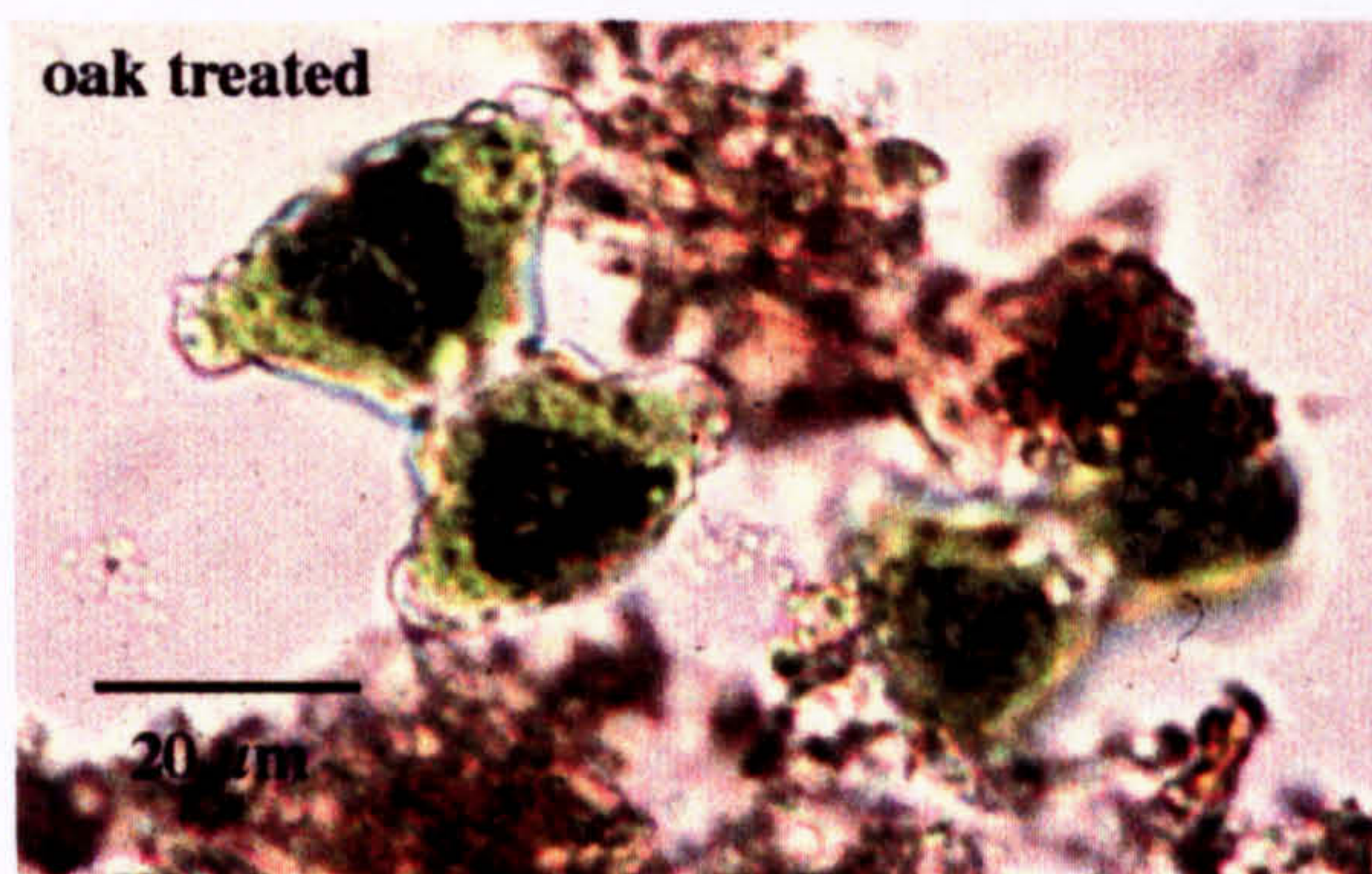
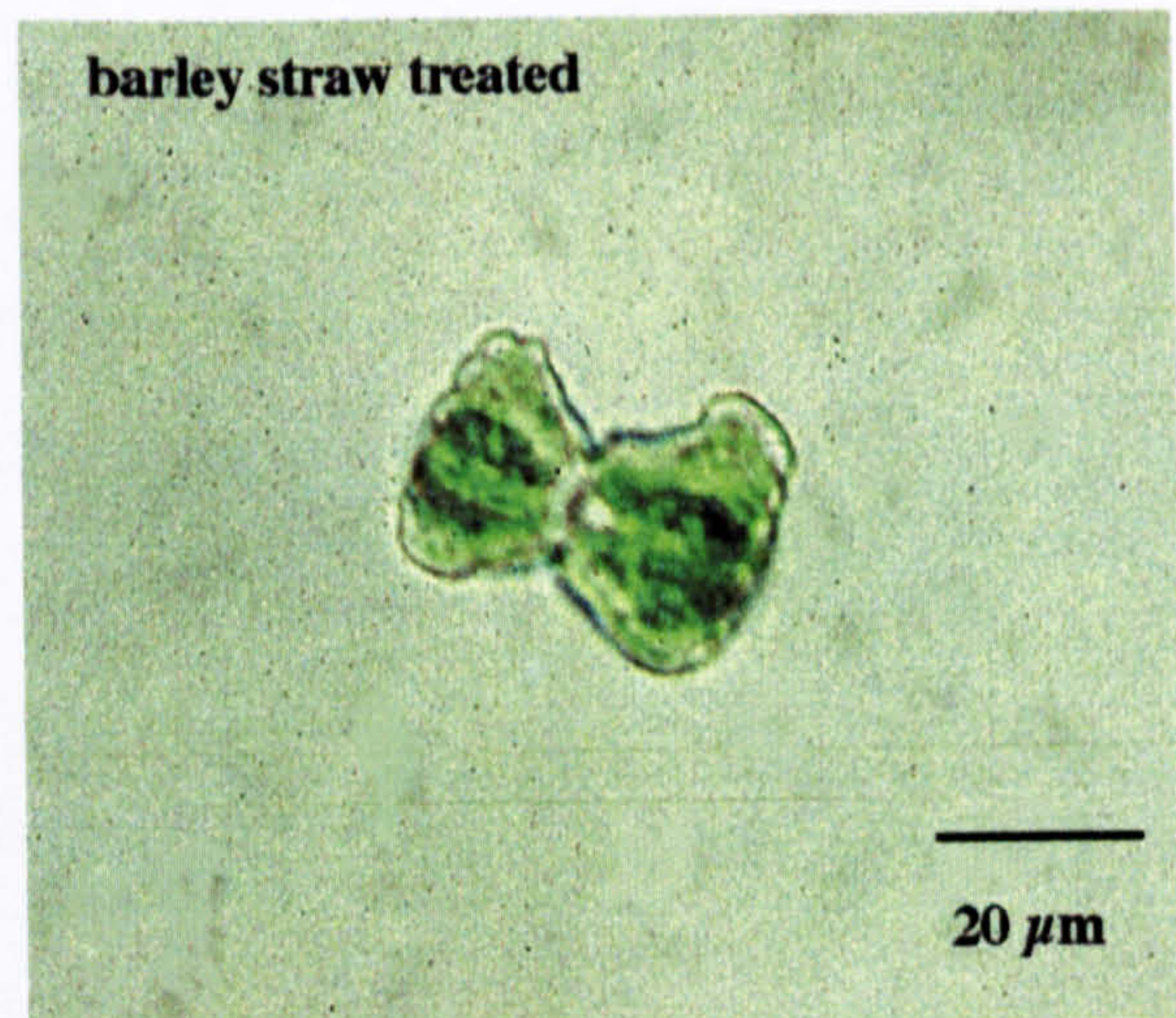
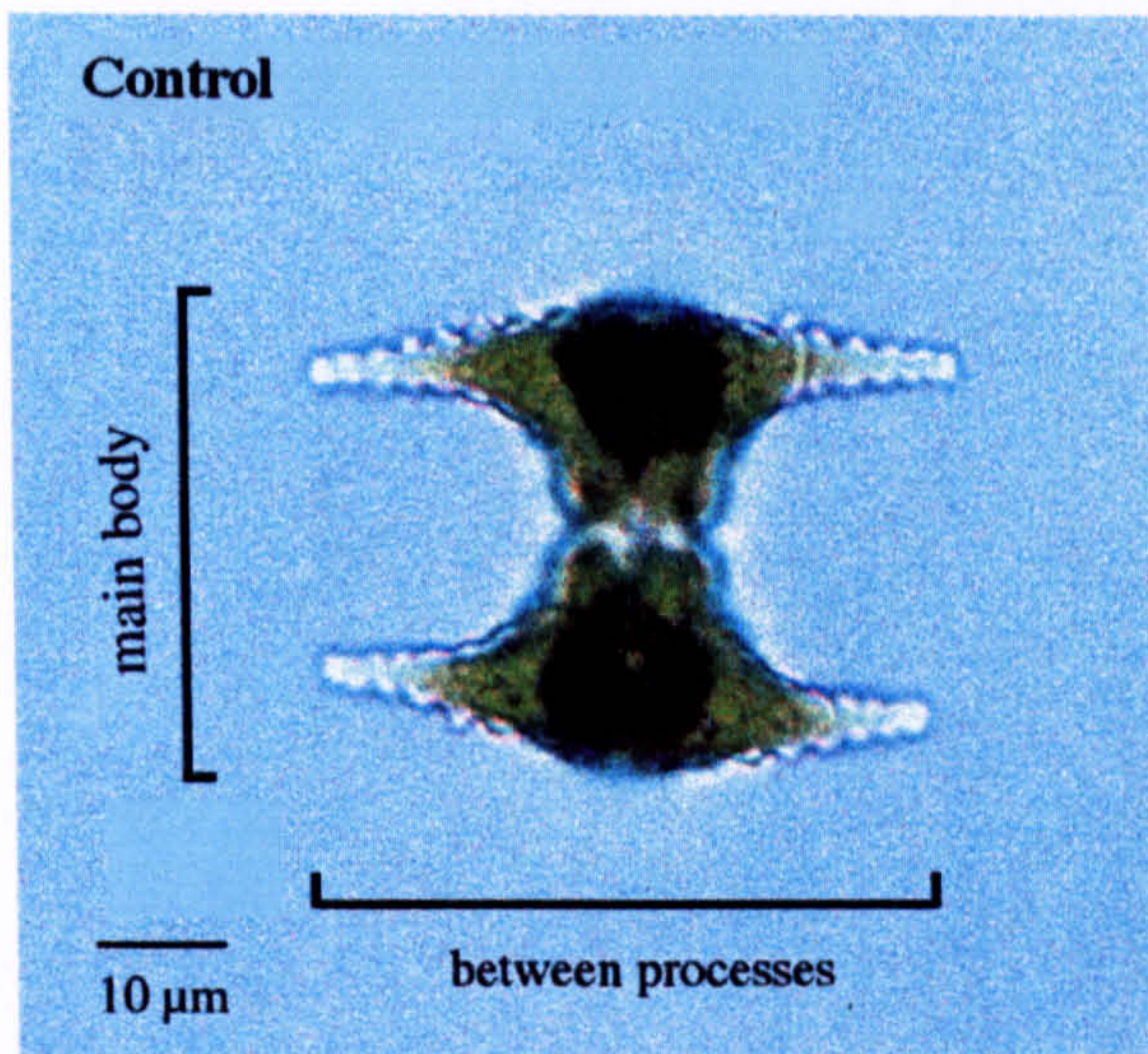


Plate 4.1 *Staurastrum pingue* after treatment with plant litter and copper sulphate.

Control image shows the measurements taken of the cell.

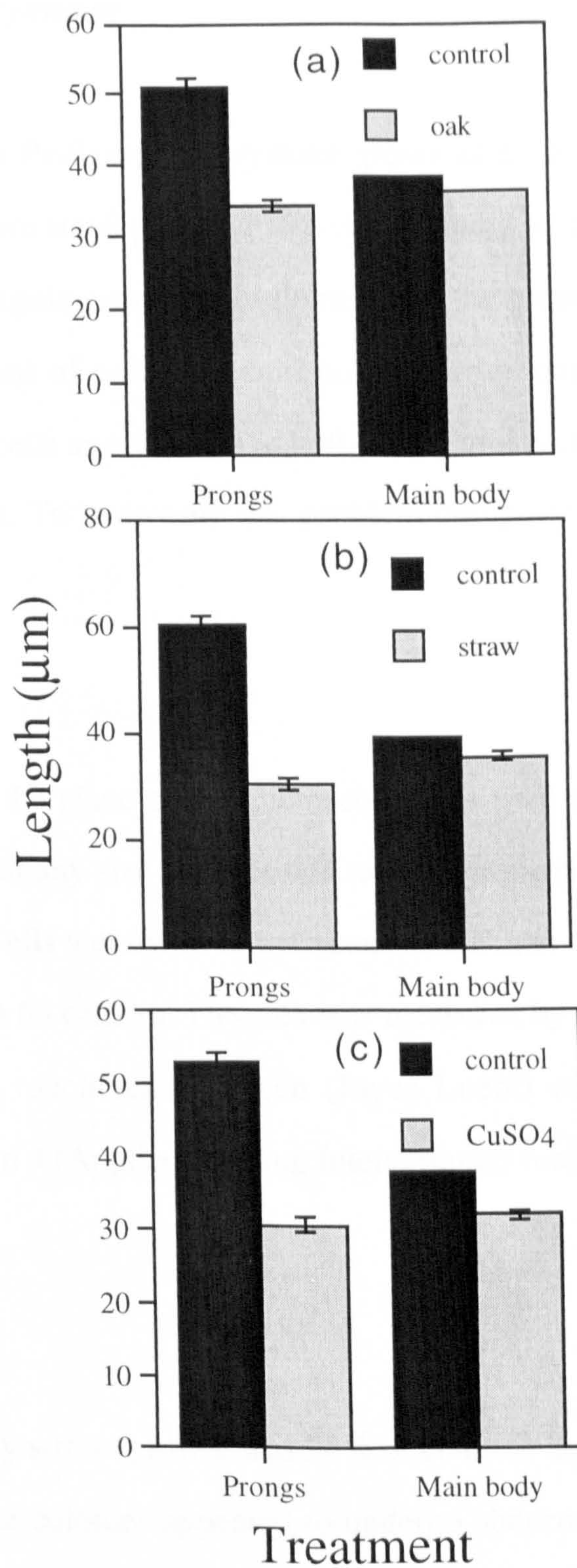


Figure 4.1 Mean length of processes and main body of cell in *Staurastrum pingue* treated with (a) 308 g m^{-3} oak leaf FPOM (b) 3636 g m^{-3} barley straw and (c) 0.02 mM copper sulphate. Values show means $\pm \text{SE}$ ($n=50$), reduction in size is significant in all cases $p < 0.001$.

growth was 17% of the control). The morphology of the cells also looked the same whether they were inhibited by plant litter or by copper sulphate (see Plate 4.1).

4.2.2 *Pediastrum boryanum*

The chlorococcalean alga *Pediastrum boryanum* grows as a flat plate of cells, the outer ones usually possessing two small prongs. From cell counting of the assays it appeared that the colony was not undergoing normal development in the presence of plant litter (Plate 4.2). Further investigations of cell size could not be carried out easily by using an eyepiece graticule since the cells and colonies in both the control and treated cultures were not simple geometric shapes. To overcome this problem computer-aided measurement was used.

Cell size measurement

Cell area, as seen under the plane of the microscope, was used as an estimate of cell size since it was presumed that any size changes due to the treatments would be reflected in all dimensions of the cell. Cells were viewed by means of a Nikon microphot-FX microscope connected to a Mti series 68 camera. The area was measured by drawing around cells on a monitor screen using a touch sensitive pen (Joyce Loeb) which was connected to a Magiscan analysis system 4 (Applied Imaging International) running genias 4.4 software.

Results

When treated with barley straw (growth was 24% of control) and copper sulphate (growth was 15% of control), the colonies appeared to undergo abnormal development, since the prongs of the outer cells did not develop or there were fewer of them (Plate 4.2). However, the cell size (measured as cell area) was not significantly different from the control with either barley straw or copper sulphate treatment (Figure 4.2).

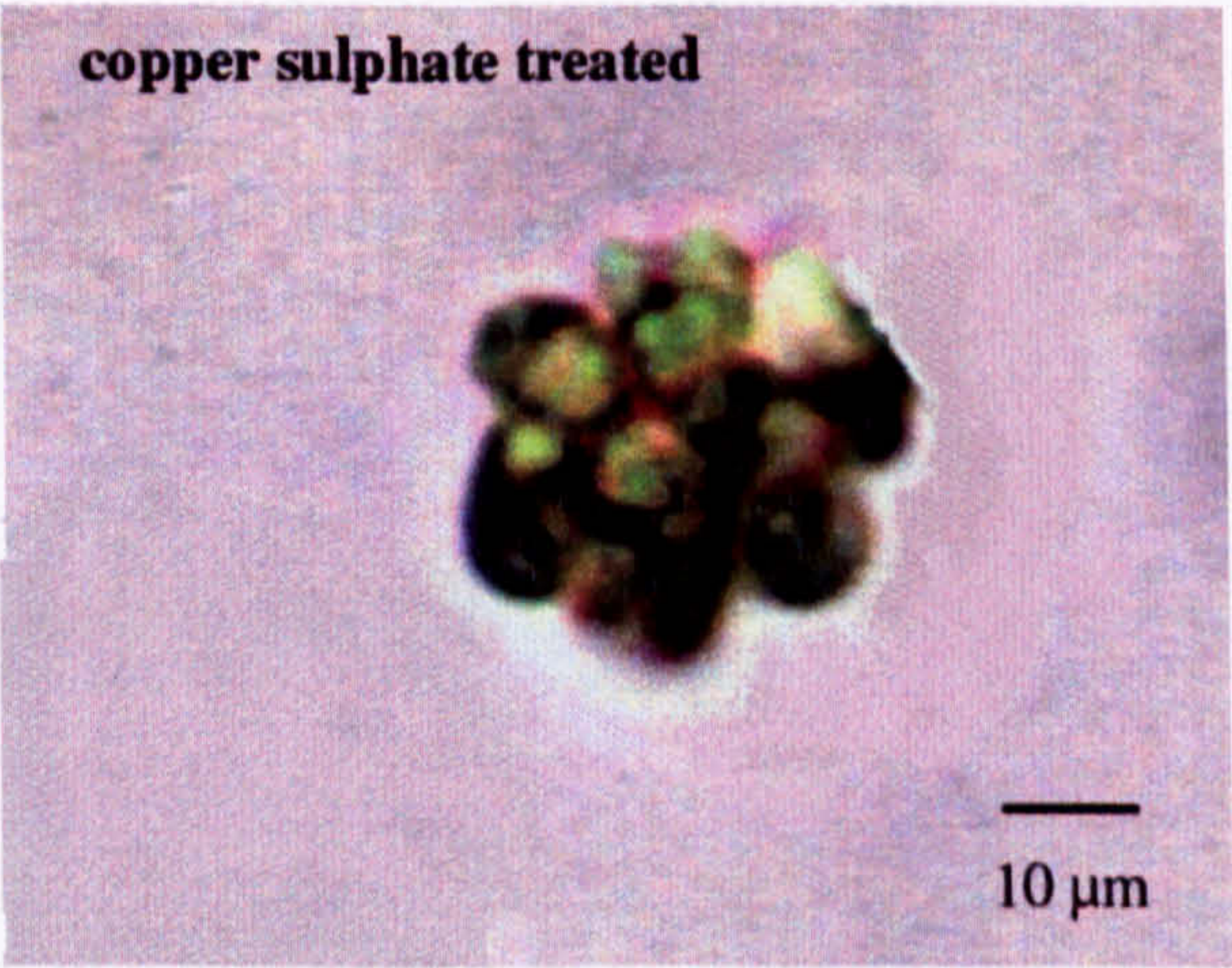
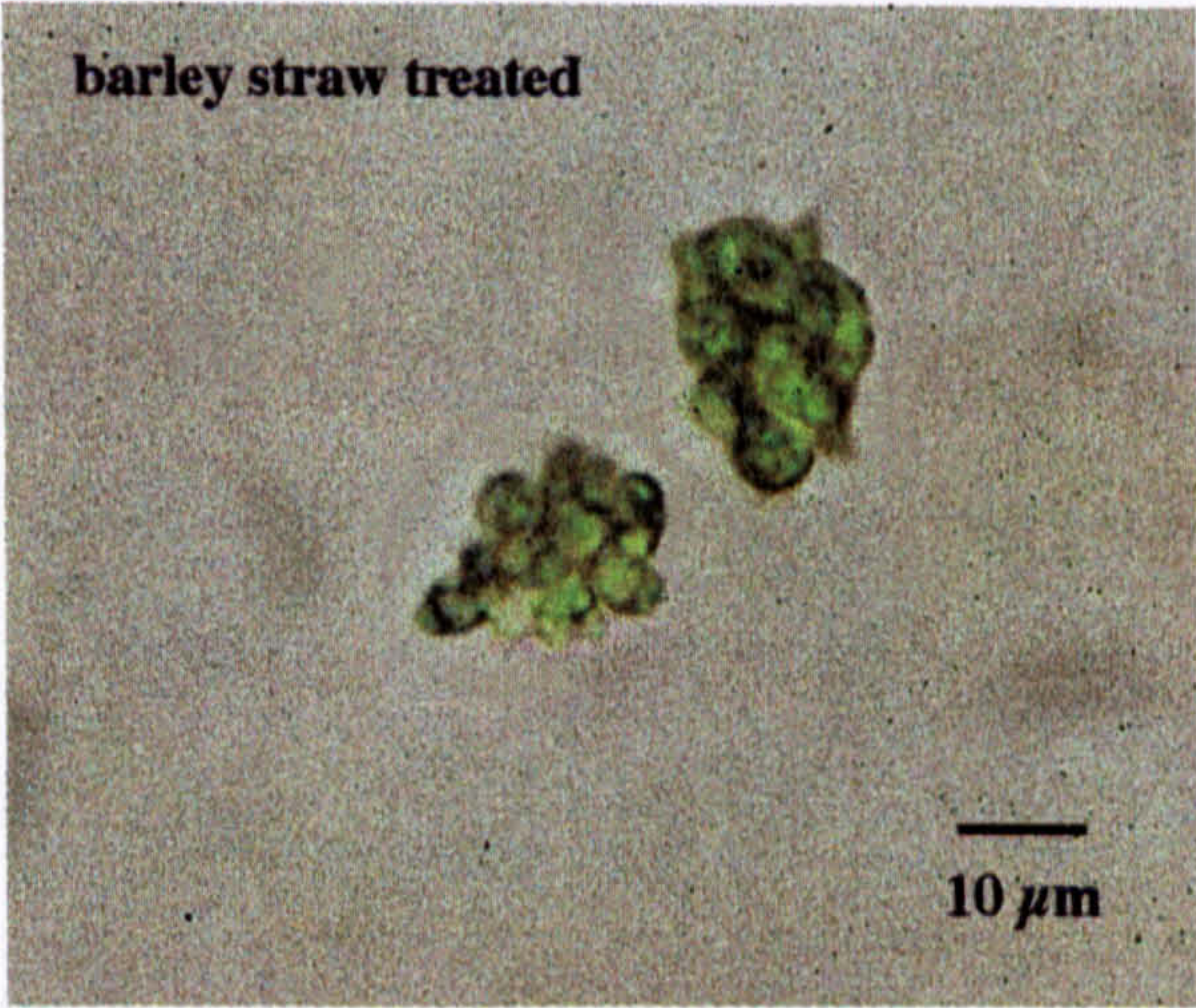
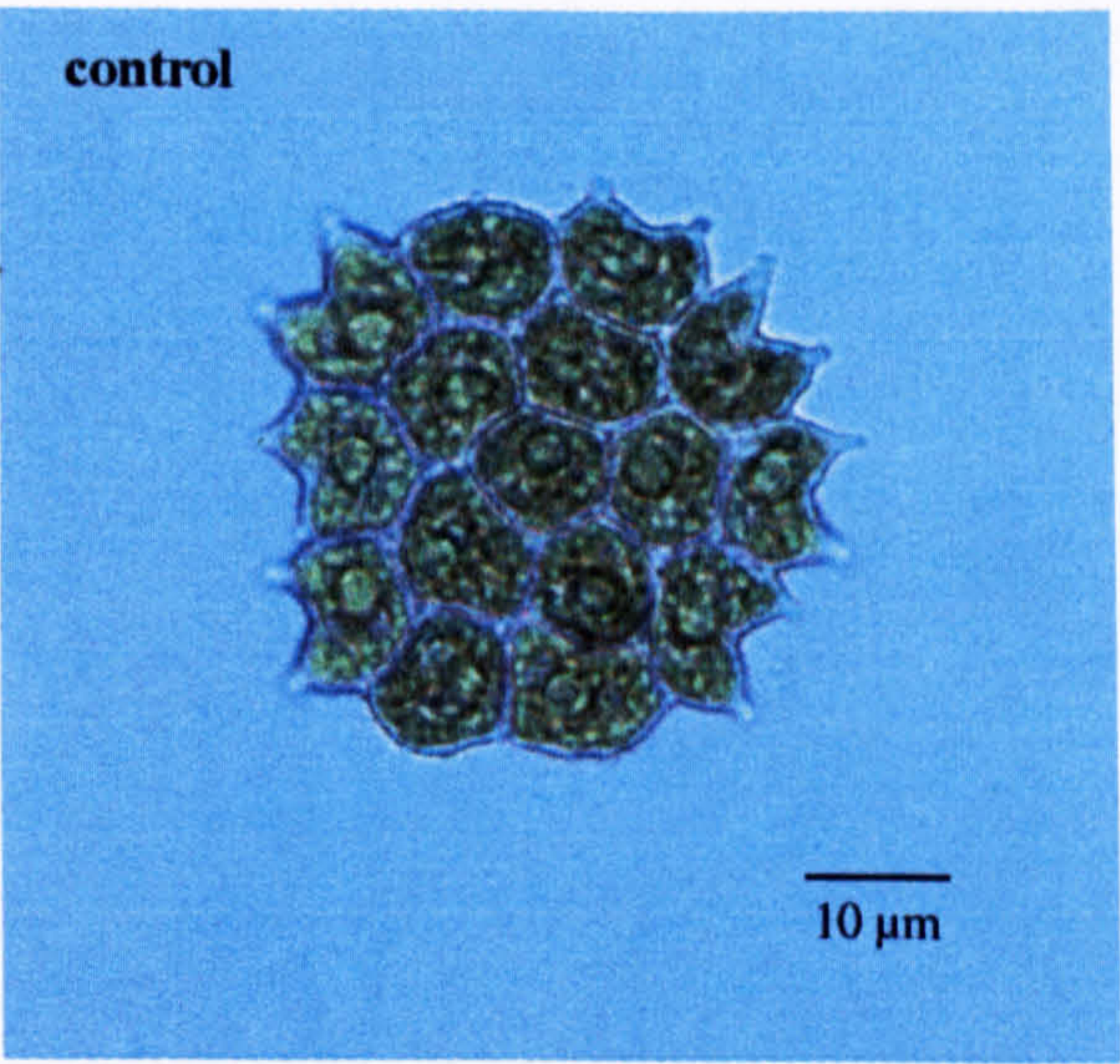


Plate 4.2 *Pediastrum boryanum* after treatment with plant litter and copper sulphate.

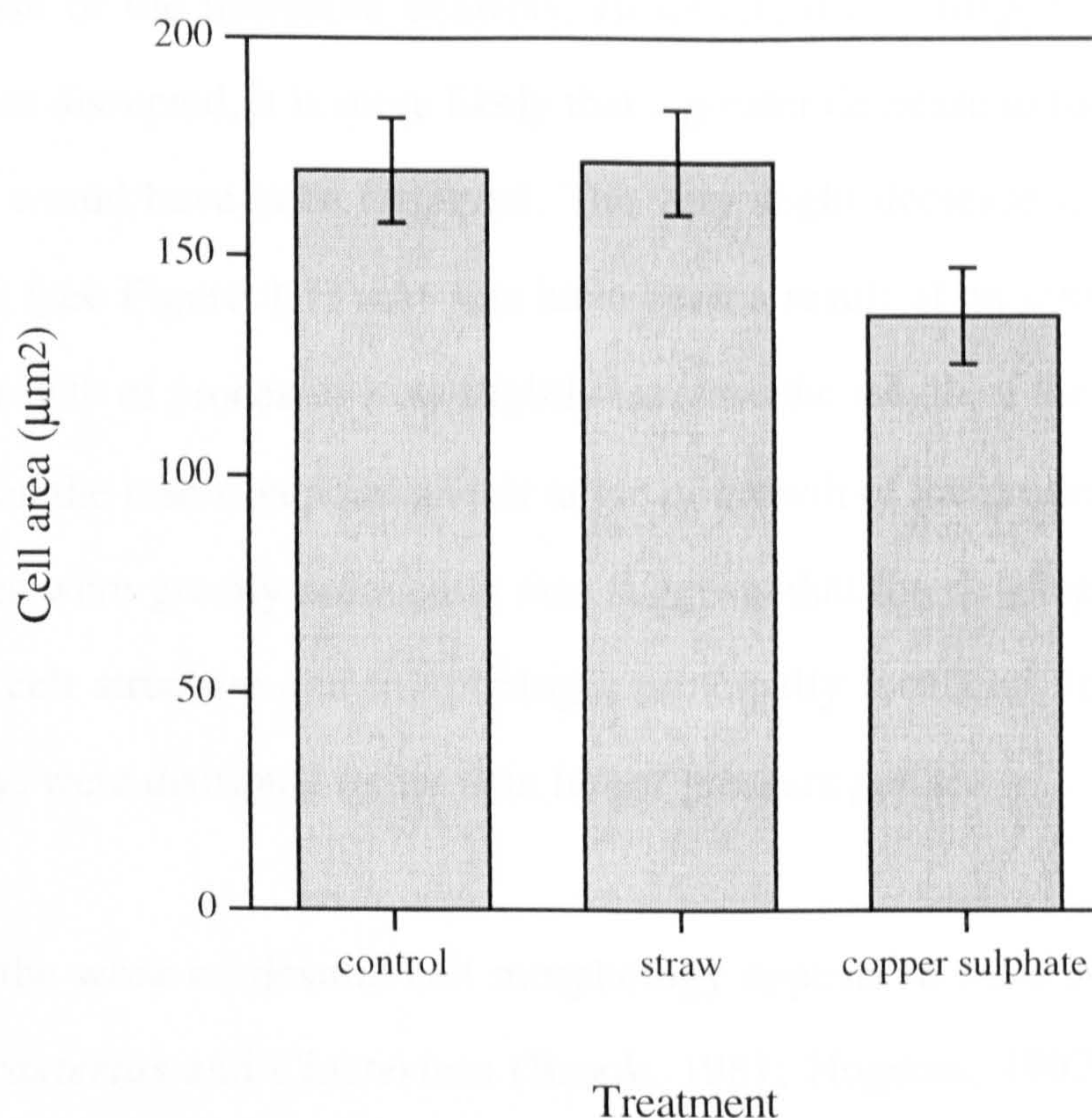


Figure 4.2 Mean cell area of *Pediastrum boryanum* when treated with 2727 g m⁻³ dry mass of barley straw and 0.02 mM copper sulphate. Values shown are means \pm SE (n=50). Areas are not significantly different.

4.2.3 Discussion

Stauroastrum pingue

The change in cell size is apparent in *Stauroastrum pingue* with both types of plant litter and with copper sulphate. The reduction in cell size may be related to growth rate since a significant negative correlation has been found between cell volume and growth rate in a number of *Stauroastrum* species (Gerrath, 1993; Coesel & Wardenaar, 1994). The current results (Figure 4.1, Plate 4.1) show that the bulk of cell volume reduction appears to be in the growth of the cell processes. Whether this was the case in previous work showing volume reductions (Coesel & Wardenaar, 1994) is not known.

Turgor pressure has been shown to be the principal process driving expansion of new semicells since a reduction in cell turgor disrupts the shape of *Micrasterias* (Kiermayer, 1981). The inhibitors used in the present investigation could have disrupted cell turgor since the main body of the cells of *S. pingue* were slightly, but significantly,

smaller than those of the untreated controls. However, if cell turgor was the principal process which was disrupted, it is more likely that a greater decrease in the size of the main body of the cell would have been observed. The very slight decrease in size of the main body of the cells (see Figure 4.1) may just have been a result of an inhibition of process growth i.e. the growth of processes may slightly increase the length of the main body of the cells depending on the insertion point and/or angle of growth of the processes. The fact that the cell processes were greatly reduced in size suggests that the developmental processes which underlay cell structure and morphology, principally localized tip growth and cell wall extensibility, were disrupted rather than turgor pressure *per se*.

Most of the work on desmid cell morphology appears to have been conducted on species of *Micrasterias* and *Closterium* (Brook, 1981; Hogetsu, 1992; Gerrath, 1993). Comparisons between *S. pingue* and *Micrasterias* can be made since they have the same type of cell division and both taxa develop cell processes or lobes (Brook, 1981). In *Micrasterias* a pre-pattern for the later cell shape is thought to be present at the plasma membrane during the early stages of septum formation (Brook, 1981; Meindl, 1993). The pre-pattern is realized by the local incorporation of cell wall material, delivered by vesicles which are thought to be directed to sites of high ion (possibly calcium) accumulations (Meindl *et al.*, 1992; Meindl, 1993).

The traditional view of development in *Micrasterias* suggests that lobe expansion is mediated by bundles of actin filaments which terminate at the tips of the growing lobes; the opposite ends of these filament bundles being located in the lobes of the corresponding side of the mother semicell (Hogetsu, 1992). The actin filaments are thought to guide secretory vesicles to the growing tips, where enhanced extension of the cell wall and incorporation of new wall material expands the lobes, or processes in the case of *S. pingue*. More recent work has failed to provide support for actin being used as a template for polarized growth since it was found not to be present in the growing lobes of *Micrasterias*, although it was present in other parts of the cell (Meindl *et al.*, 1994). However, the presence of actin in growing lobes cannot be ruled out since the actin in growing lobes may

be different to that in the remainder of the cell; actin may not easily be visualized with the fluorescent dyes used up to the present (Menzel, 1996). Menzel (1996) suggested that lobe growth in *Micrasterias* may be completely governed by processes acting at the time of pre-pattern formation in the plasma membrane (see above). He speculated that the membrane may possess different types of docking protein, accepting vesicles carrying either material for wall expansion or material designed to stiffen the wall. In this way different areas of the wall would grow or cease growing to give the cell its characteristic shape. This hypothesis could also explain the lack of actin found in growing lobes, since pattern formation could be so short-lived and involve such small amounts of actin that it has evaded attempts to visualize it.

Pediastrum boryanum

In *Pediastrum boryanum* zoospores released from the parent cell are contained inside a vesicle, within which they arrange themselves to form a complete daughter colony (see section 3.3.1). When treated with barley straw or copper sulphate the cells in the daughter colony expand normally, but few of the cells appear to have the prongs which are typically associated with the peripheral cells (Plate 4.2 & Figure 4.2); the structure of the colony also appears to be disrupted. Zoospores are released in an ovoid to spherical form (Marchant, 1979) with the arrangement of the cells in the new colony being fixed before the zoospores retract their flagella (Marchant & Pickett-Heaps, 1974). All of the cells in a colony of *P. boryanum* have at least four prong sites (occurring in pairs both dorsally and ventrally) but the tight adhesion of the inner cells after the colony is assembled suppresses prong extension, leaving only the outer cells free to produce prongs on their outward facing surfaces (Millington *et al.*, 1981). The inhibitors may therefore be acting upon a cellular mechanism that occurs during zoospore formation or immediately after zoospore release, which disrupts cell and colony development but which does not affect overall cell expansion.

The capacity of the prongs to extend can vary depending on environmental conditions such as pressure and the osmotic strength of the surrounding medium (Millington *et al.*, 1981). As with *S. pingue*, environmental conditions are unlikely to play any part in cell deformation in *P. boryanum* since all the cells were grown under the same conditions. Disruption is more likely to result from processes which control cell morphology. In *P. boryanum* cell shape and prong formation are thought to be regulated by microtubules which co-ordinate the expansion and deposition of new wall material (Marchant & Pickett-Heaps, 1974; Marchant, 1979). These microtubules have a cytoskeletal function, encircling the zoospore just beneath the plasma membrane and extending into the prongs (Marchant & Pickett-Heaps, 1974; Millington & Gawlik, 1975). The microtubules arise from microtubule organising centres, the disruption of which results in shorter and more variable numbers of prongs on cells (Marchant, 1979).

Conclusions

Overall it appears that the underlying effect of both copper sulphate and the litter-derived inhibitors is on the controlling mechanisms of cell morphology. In the presence of the litter-derived inhibitors algal cells appear to expand normally (shown by the fact that no other inhibited species exhibited smaller cells, apart from *S. pingue* (see above)) but in the more elaborate cell shapes of *S. pingue* and *P. boryanum* the growth of 'prongs' is impaired. It is known that some chemicals can disrupt microtubules causing the disorganisation of new cell wall microfibrils but while still allowing deposition of new cell wall material (and therefore expansion) to occur (Brett & Waldron, 1996). Whether the altered cell morphology is due to the inhibitors affecting directly the production and/or arrangement of microtubules or filaments is not known, but in the case of copper sulphate a direct effect would appear unlikely. Copper is known to disrupt many cellular processes which could indirectly produce defects in cell morphology (McBrien & Hassall, 1965; Woolhouse, 1983; Fernandes & Henriques, 1991). It would appear more likely that inhibition is acting indirectly via, as yet, unidentified process(es).

4.3 Changes in colony size

A number of the species tested were colonial algae two of which, *Pediastrum boryanum* and *Asterionella formosa*, appeared to show a reduced number of cells per colony (reduction in colony size) when grown in the presence of the plant litter-derived inhibitor(s). This apparent reduction in cells per colony was compared with growth in the presence of the general growth inhibitor copper sulphate.

4.3.1 *Pediastrum boryanum*

Results

It is apparent from Figure 4.3 that the number of cells in a colony of *Pediastrum boryanum* was significantly reduced in the presence of decomposed barley straw (overall growth was 24% of the control). This reduction in colony size was also seen when *P. boryanum* was treated with copper sulphate (overall growth was 15% of the control). The reduction in cells per colony appears greater with the copper sulphate, which also shows a greater overall inhibition in cell numbers.

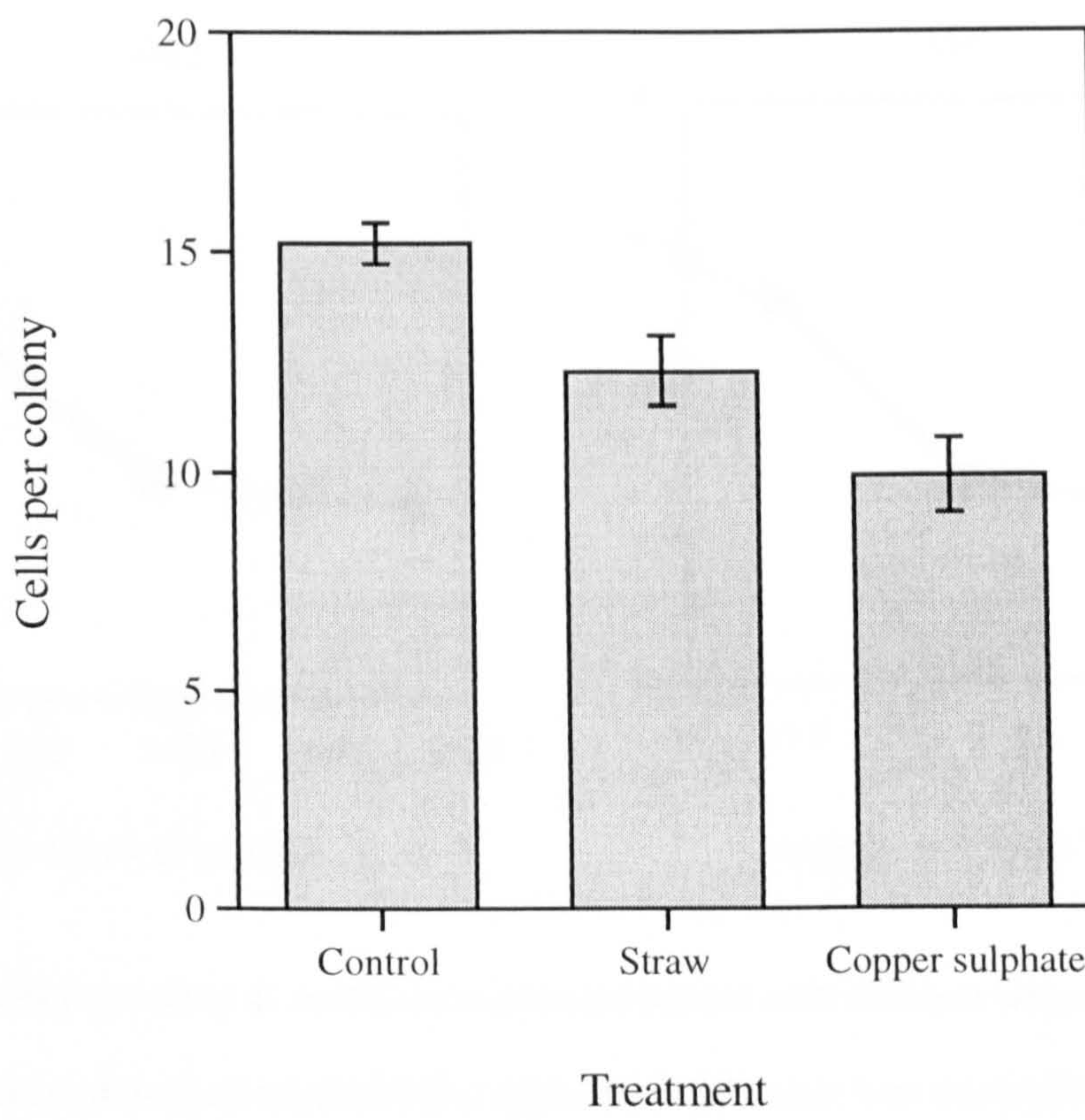


Figure 4.3 Mean cells per colony in *Pediastrum boryanum* treated with 0.02 mM copper sulphate and 2727 g m⁻³ dry mass barley straw. Values are means ±SE (n=50). Reduction in mean cells per colony with both inhibitory compounds is significant p <0.001.

4.3.2 *Asterionella formosa*

Results

There was clearly a reduction in cells per colony in *Asterionella formosa* when inhibited with decomposed barley straw or with copper sulphate (Figure 4.4). At the highest levels of inhibition only two cells per colony were seen as oppose to six when *A. formosa* was growing uninhibited. The number of cells per colony is a linear function of the amount of inhibition (Figure 4.5). Comparison of regressions showed there was no significant difference in the relationship between growth and cells per colony with copper sulphate and barley straw (p >0.3).

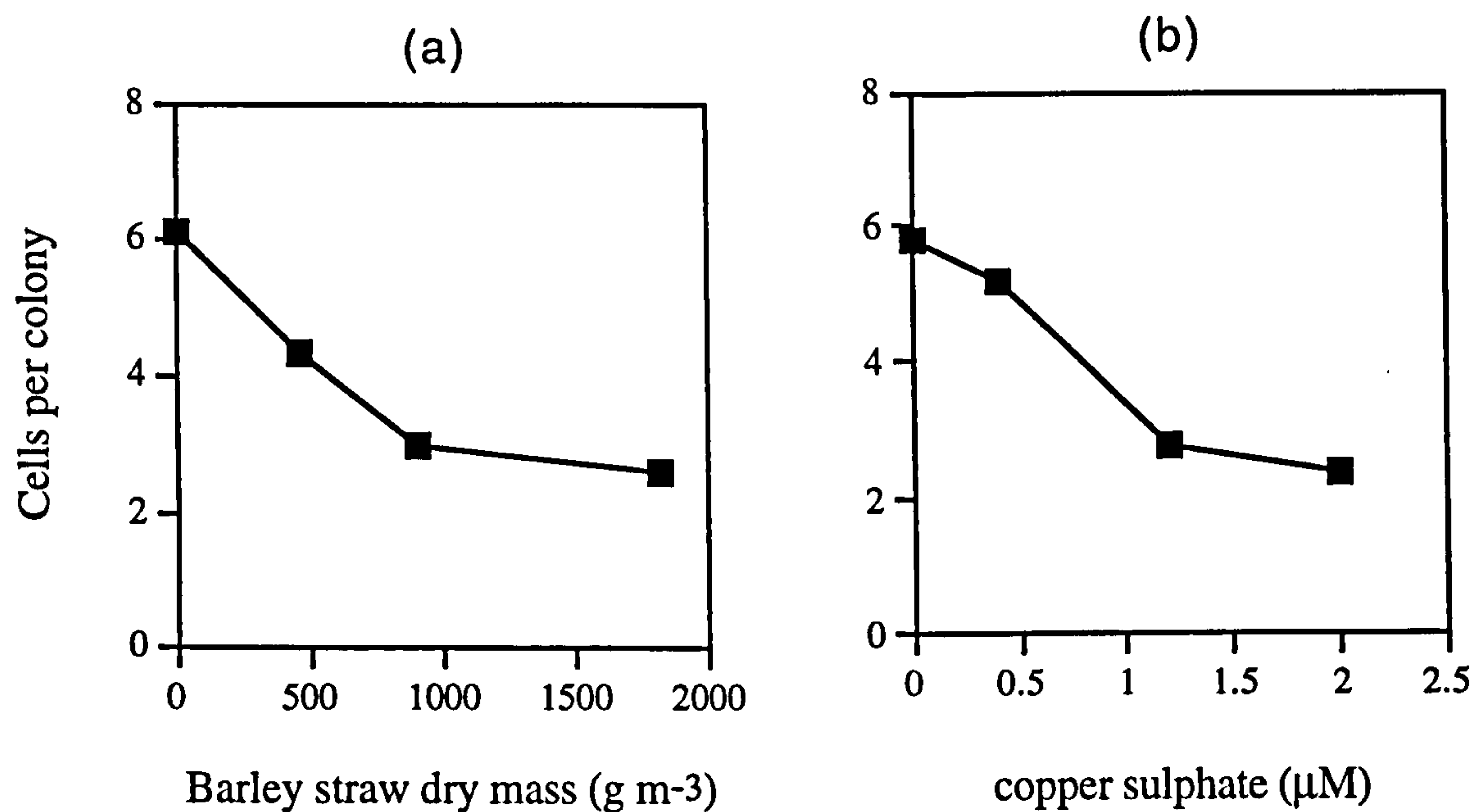


Figure 4.4 Mean cells per colony in *Asterionella formosa* treated with different masses of barley straw and copper sulphate. Values shown are means of five replicates \pm SE (error bars do not show where error is too small). Decrease in cells per colony is significant for both barley straw and copper sulphate $p < 0.001$.

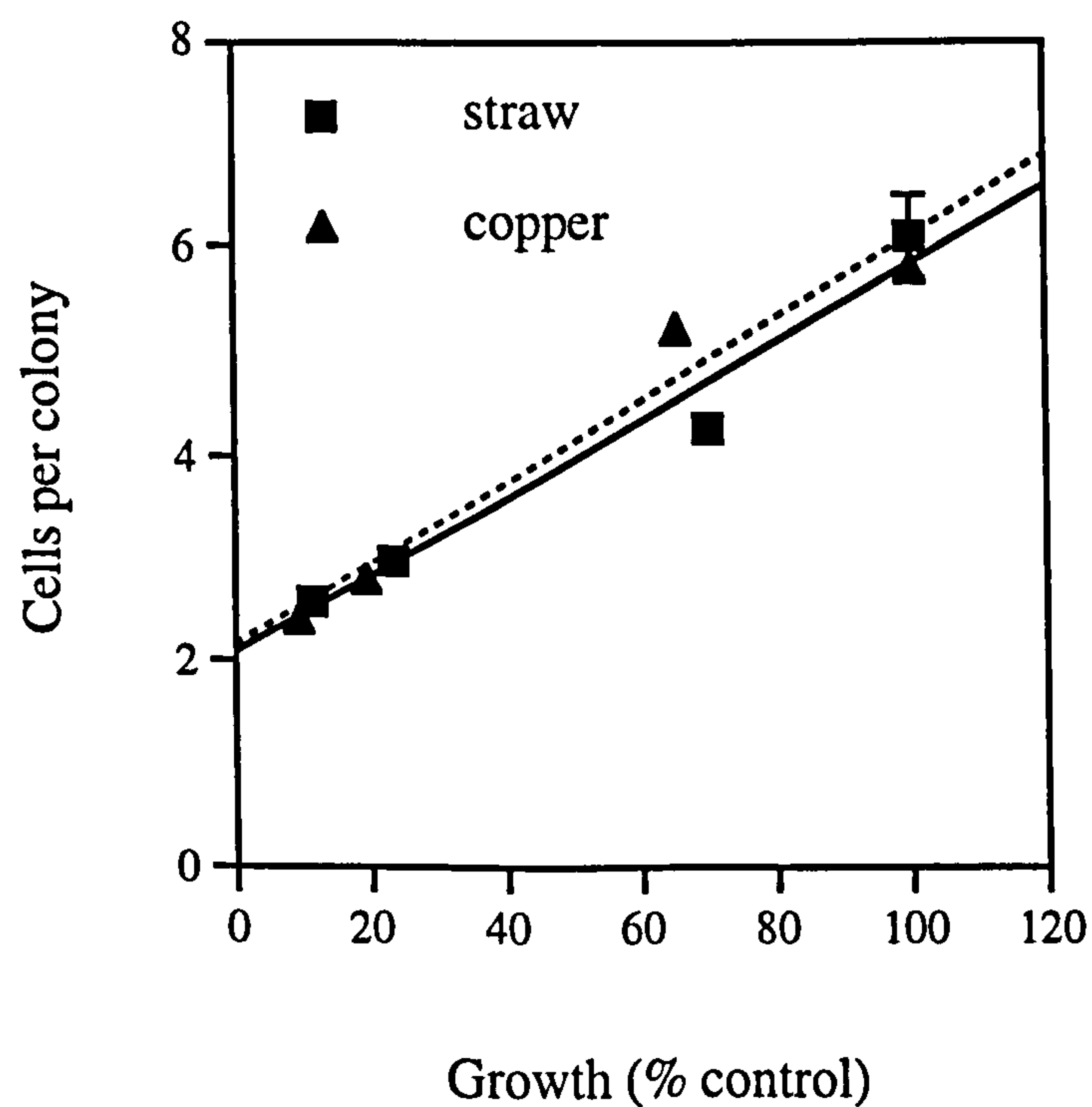


Figure 4.5 Number of cells per colony in *Asterionella formosa* plotted against growth as a percentage of the control value. Values are means ($n=5$) \pm SE (errors too small where bars not shown). Regression with straw (solid line) $y=0.037x + 2.091$, $r^2=0.97$; regression with copper sulphate (dashed line) $y=0.039x + 2.147$, $r^2=0.96$.

4.3.3 Discussion

In *Asterionella formosa* the number of cells per colony is known to change with the seasons (Lund *et al.*, 1963; Hayakawa *et al.*, 1994 and references therein) and with varying nutrient regimes (Tilman *et al.*, 1976). The effect of temperature on the number of cells per colony was investigated by Hayakawa *et al.* (1994) who found that the number of cells per colony is related to the specific growth rate rather than temperature *per se*. The results obtained in the present investigation are analogous to those of Hayakawa and co-workers, since the reduction in the number of cells per colony obtained with barley straw and copper sulphate was dependant on the overall growth inhibition (see Figure 4.5).

A. formosa cells are connected to each other by means of mucilage pads at the base of the cells (Bellinger, 1992; Hayakawa *et al.*, 1994; Plate 3.1). It is probable that the cell connections can be controlled by the amounts, freshness, or chemical structure of the mucilage connections. Whether the direct production of the specific number of cells per colony, rather than the division of larger colonies, was the favoured mechanism of colony production is not known. Further detailed investigations measuring colony size at different time intervals would have to be carried out to investigate this further. Such investigations may also provide evidence as to whether colony size reduction is due to weakening of the mucilage and purely physical break up of the colonies, or whether reduction in colony size is a developmental process. From previous investigations and the results shown here it appears that no specific effect causes the reduction in cells per colony with decreased growth rate. The comparison with temperature in particular, suggests that effects on entire cellular metabolic processes are important especially those which may influence cellular connections.

In *Pediastrum* species the cell number in a colony is determined by the number of mitoses in the parental cell (Millington & Gawlik, 1975), fewer mitoses produce fewer zoospores and, therefore, there are fewer cells in a colony. Figure 4.3 shows that there was a reduction in the number of cells in a colony of *P. boryanum* when grown under the

influence of copper sulphate or barley straw. Consequently, *P. boryanum* must undergo a reduced number of mitoses in the parental cells, or the connections between cells may be weakened allowing cells to break off easily from the colony. As is seen with *A. formosa*, the reduced number of cells per colony may be due to a decreased growth rate, since the greatest decrease in cells per colony occurred with copper sulphate where, in this case, overall inhibition of growth was strongest. Further investigations with a range of concentrations of barley straw and copper sulphate are required for a better comparison.

The overall conclusion must be that the effects on colony size are not a specific effect of litter-derived inhibitor(s) but they are a general effect arising from a reduction in growth rate.

4.4 Ultrastructural investigations (electron microscopy)

Most of the species that were assayed showed no structural or morphological changes in the presence of litter-derived inhibitors. However, when *Scenedesmus quadricauda* was grown with barley straw the amount of chlorophyll *a* per cell was significantly reduced. The reduction in chlorophyll *a* per cell was observed both when growth was inhibited and when there were no significant differences in total cell numbers between straw-treated and control cultures (Figure 3.12). Thus, cultures of *S. quadricauda* treated with barley straw appeared to produce the same yield of cells as control cultures, yet the treated cells contained only one quarter to one third the amount of chlorophyll *a* as the control cells.

It appears paradoxical that equivalent yields of cells can be achieved when the major light harvesting pigment (i.e. chlorophyll *a*) is reduced by so much in barley straw-treated cultures. It is possible that control cultures could be producing chlorophyll *a* which is not used in photosynthesis (i.e. overproducing chlorophyll *a*); this would mean straw-treated cultures could obtain equivalent yields of cells, but with less chlorophyll *a*. However, this is very unlikely since the control cells would have had to produce three

times more chlorophyll *a* than was being utilized during photosynthesis, which physiologically and energetically is uncompetitive. Barley-treated cultures of *S. quadricauda* may have switched to the production of other light-harvesting pigments to off-set the loss of chlorophyll *a*. However, no different peaks of absorption of a pigment extract (using methanol-see section 2.3.3.2) were observed between straw treated and control cultures at light wavelengths between 380-700 nm (results not shown).

Under the light microscope, cells of *S. quadricauda* appeared to be more 'granular' when treated with barley straw but further investigations were hampered by the lack of magnification. Using electron microscopy, ultrastructural changes in the cells that were not apparent under the light microscope could be determined. It was hoped that electron microscopy would also give some clues as to how the cells were growing with less chlorophyll *a*.

Electron microscopical investigations were also carried out on both *Anabaena flos-aquae* and *Anabaena cylindrica*. *A. flos-aquae* had been shown to be inhibited by barley straw (see section 3.6.2) so ultrastructural investigations were carried out to ascertain the effects of barley straw. *A. cylindrica* was stimulated in the presence of straw, therefore electron microscopy could be used to see if stimulated cells differed to those from control cultures; a comparison between the two members of the same genus could also be made.

4.4.1 Methods

Cultures of all the species to undergo electron microscopy were prepared according to the assays used in Chapter 3 (see section 2.3 for methods), using five flasks for each treatment. *A. flos-aquae* and *A. cylindrica* were grown for eight days with 700 and 1800 g dry mass barley straw m⁻³ medium respectively. *S. quadricauda* CCAP 276/21 was grown with 2000 g dry mass barley straw m⁻³ medium for four days (to try and obtain cultures where cell numbers were inhibited in the early stages of growth) and eleven days (to try and obtain cultures after a recovery in cell number).

In all cases, one flask for each treatment was used for electron microscopy while the other flasks were used to estimate biomass (see Table 2.6) so comparisons of growth between treated and control cultures could be made.

4.4.1.1 Fixation

All the chemicals were supplied by Agar Scientific unless otherwise stated. For both *Anabaena* species the cells were fixed for 24 hours at room temperature in 2.5% glutaraldehyde made up in the growth medium the cells were grown in (JM1). Cells of *S. quadricauda* were fixed for 24 hours at room temperature in 2.5% glutaraldehyde and 2% paraformaldehyde made up in the growth medium the cells were grown in (JM1). After fixation, the protocols for each species were identical.

Fixed cultures were centrifuged at 2000 g (3000 rpm) on a bench-top centrifuge (Fisons) for five minutes. The pellet was resuspended in JM1 growth medium in a microcentrifuge tube (BDH) and centrifuged at 2200 g (4000 rpm) on a bench-top microcentrifuge (Corning-Costar) for two minutes. Resuspension in fresh medium and centrifugation was repeated twice more to wash the algal cells. The pellet was resuspended in 1% osmium tetroxide for 1.5 hours then washed three times with growth medium as before. The cells were then enrobed in 1% low gelling-temperature agar (Sigma) and centrifuged at 3300 g (6000 rpm) for two minutes. The enrobed cells and agar were cut into ca. 1 mm³ blocks and dehydrated in acetone as below:

30% acetone for 10 minutes

50% acetone for 10 minutes

70% acetone for 20 minutes

90% acetone for 20 minutes

100% acetone for 15 minutes (repeated three times)

100% acetone with a molecular sieve (to exclude traces of water) for 15 minutes

4.4.1.2 Infiltration and Embedding

The cells were embedded in epon resin which was made up with 20 ml of epon 812, 22 ml of dodecenyl succinic anhydride, 5 ml of methyl nadic anhydride and 1.4 ml of benzyldimethylamine (BDMA) which was added as a hardening accelerator.

For infiltration, the dehydrated cells were placed in a 1:1 solution of epon (minus BDMA) and acetone in airtight containers for 24 hours on a rotating mixer (Agar Scientific). While still on the rotating mixer the containers were opened to the atmosphere for 3 hours. The epon was then replaced with fresh epon (minus BDMA) and mixed for a further 3 hours. The cells were finally embedded in epon (including BDMA) in square tipped capsules and polymerized at 60°C for 24 hours.

Ultra-thin sections (ca. 60 nm) were cut using a glass knife on an ultracut microtome (Reichert-Jung) and mounted on formvar-coated '200-mesh' copper grids (Agar Scientific). The sections were stained with uranyl acetate (50 minutes at 35°C) and lead citrate (10 minutes at 20°C) in an LKB ultrastainer and examined at 80 kV on a JEM 1010 electron microscope (Joel).

4.4.2 Results

When treated with barley straw the growth of *Anabaena flos-aquae* was found to be 6% of the growth of the control. For *A. flos-aquae* (Plate 4.3) there appeared to be no obvious differences in the ultrastructure of the cells between the control and treated cultures, with the thylakoids appearing to be intact in both cases. However, in the straw-treated cells, the cell wall was more crenulated, suggesting disruption of the cell in some way. The filaments of *A. flos-aquae* also appeared to have fragmented.

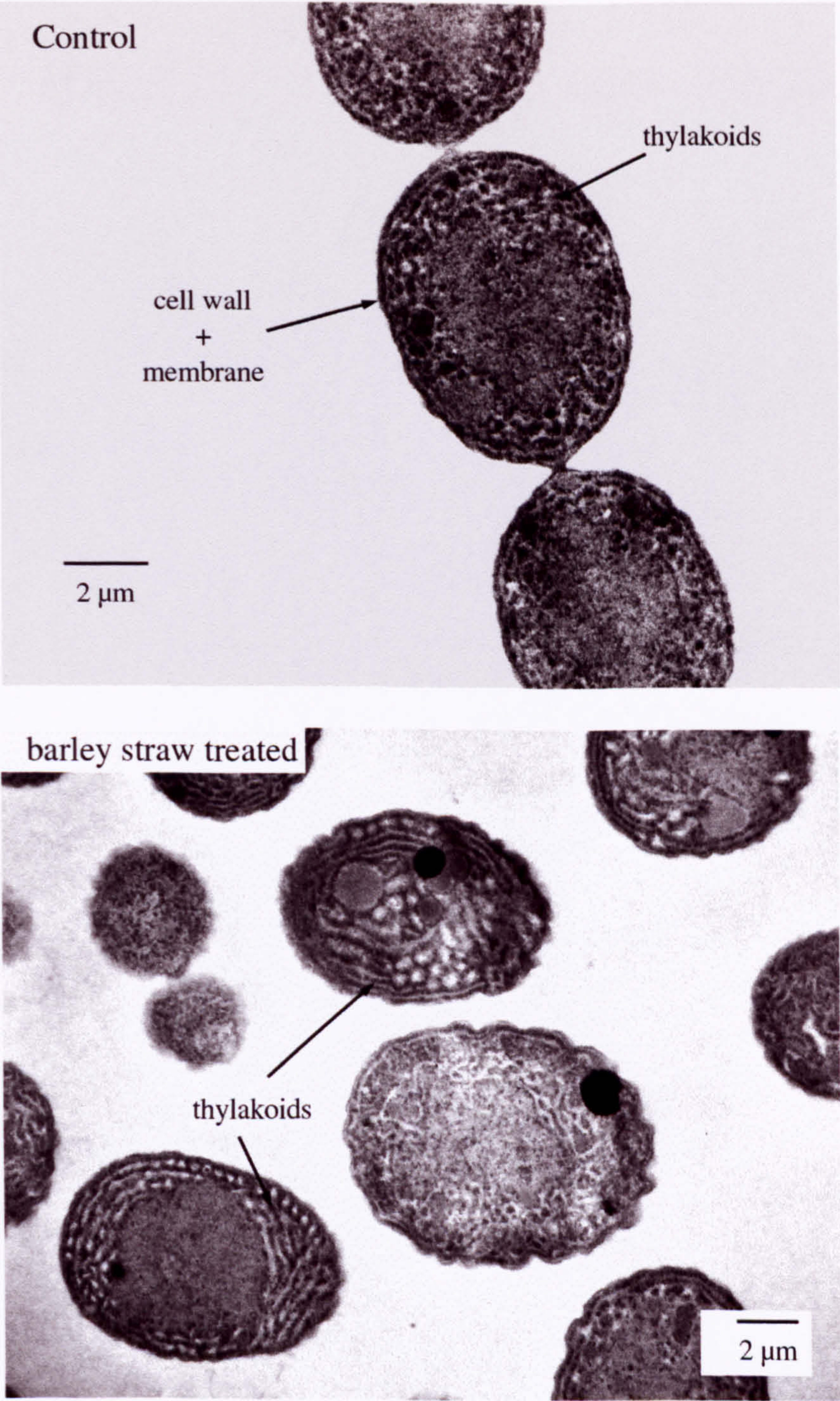


Plate 4.3 Electron micrographs of *Anabaena flos-aquae* with and without treatment with barley straw

The yield of *Anabaena cylindrica* was increased to 300% of the control value in the presence of barley straw. Plate 4.4 shows that there were no obvious differences in the ultrastructure of cells of *A. cylindrica* whether grown with or without barley straw.

The original intention was to examine cells of *Scenedesmus quadricauda* which were inhibited and ones which had recovered their cell numbers to equal or surpass those of the control (after an original inhibition of growth). However, due to the variability of *S. quadricauda* grown in the presence of straw (see section 3.3.2) no inhibition of growth was found. After four days growth, cell numbers were $213\% \pm 20$ of control values and after eleven days growth cell numbers were $226\% \pm 16$ of control values. Thus, in both cases *S. quadricauda* was stimulated in the presence of barley straw, although chlorophyll *a* was still reduced to ca. one third that of the controls. Plates 4.5 and 4.6 show that after both four and eleven days growth the cells that were treated with straw possessed many electron translucent areas. Also, when compared to the control cells, the chloroplasts were not clearly defined in the straw-treated cells and the cell contents were disrupted. There appear to be no differences between those cells grown for four days and those grown for eleven days, as was perhaps expected, since no inhibition of growth was observed in any of the cultures.

The electron translucent areas bore a resemblance to the starch sheaths which surrounded the pyrenoid (compare the starch plates of the pyrenoid with the electron translucent areas seen in the straw treated cells in Plate 4.6), although staining of unfixed live material for starch using iodine was inconclusive. Fixed and embedded 1 μm sections of the eleven day grown culture were stained with iodine to test for starch both before and after digestion with a 1% solution of α -amylase (the α -amylase was placed on sections and incubated for five minutes at room temperature). Plate 4.7 shows that the straw-treated cells had blue black inclusions in them when stained with iodine which were not present after incubation with α -amylase, showing that these areas are starch.

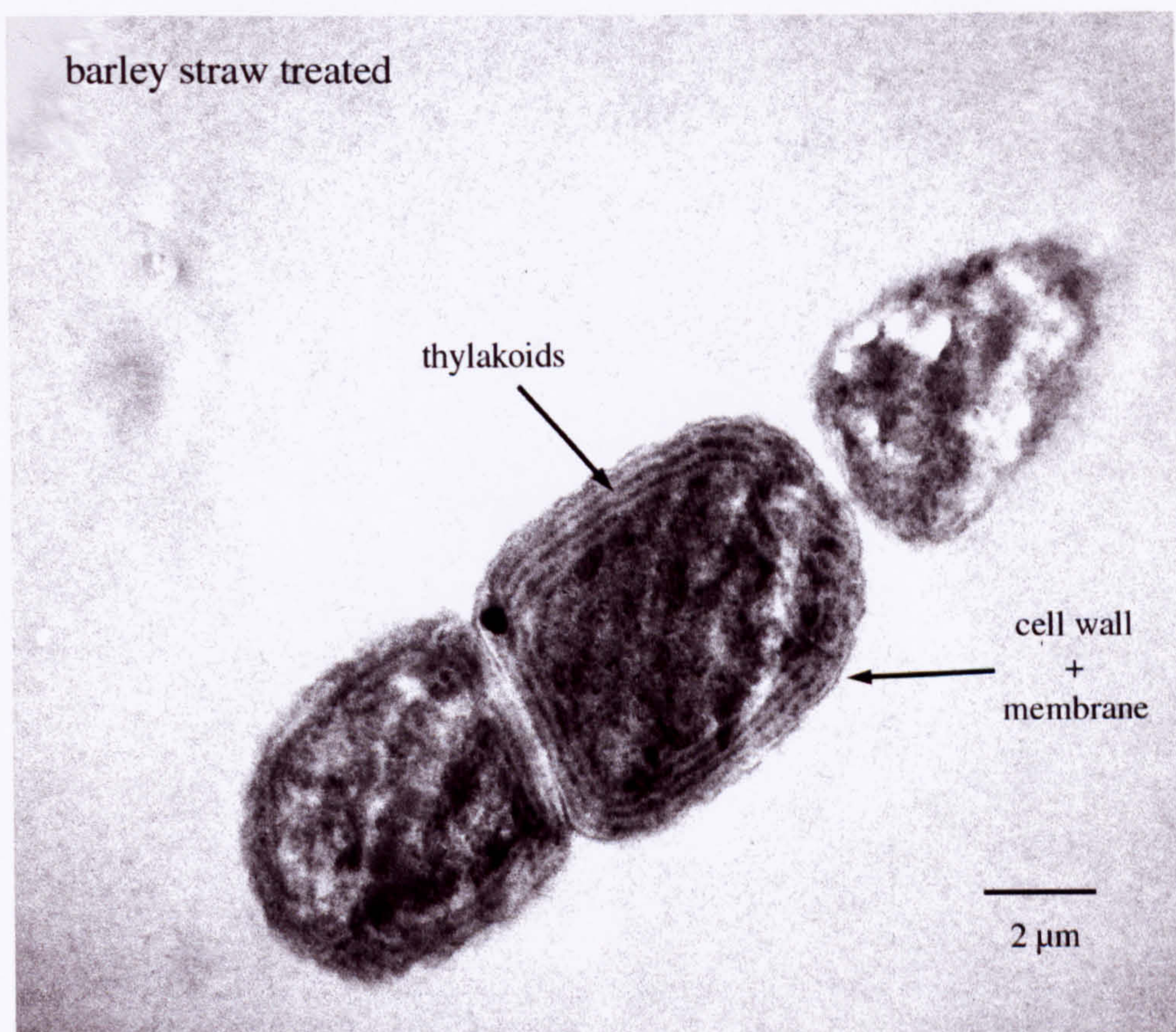
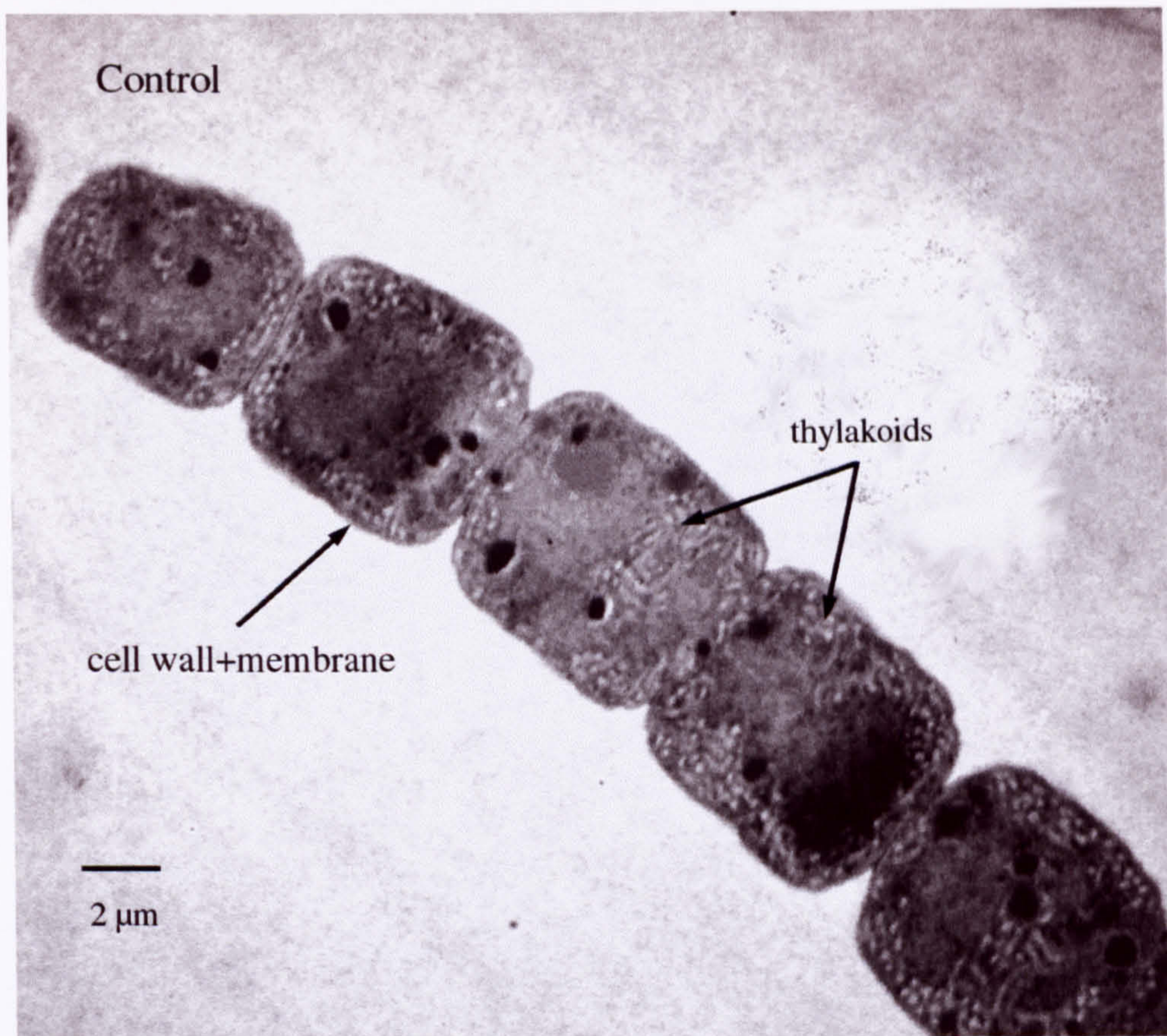


Plate 4.4 Electron micrographs of *Anabaena cylindrica* with and without treatment with barley straw.

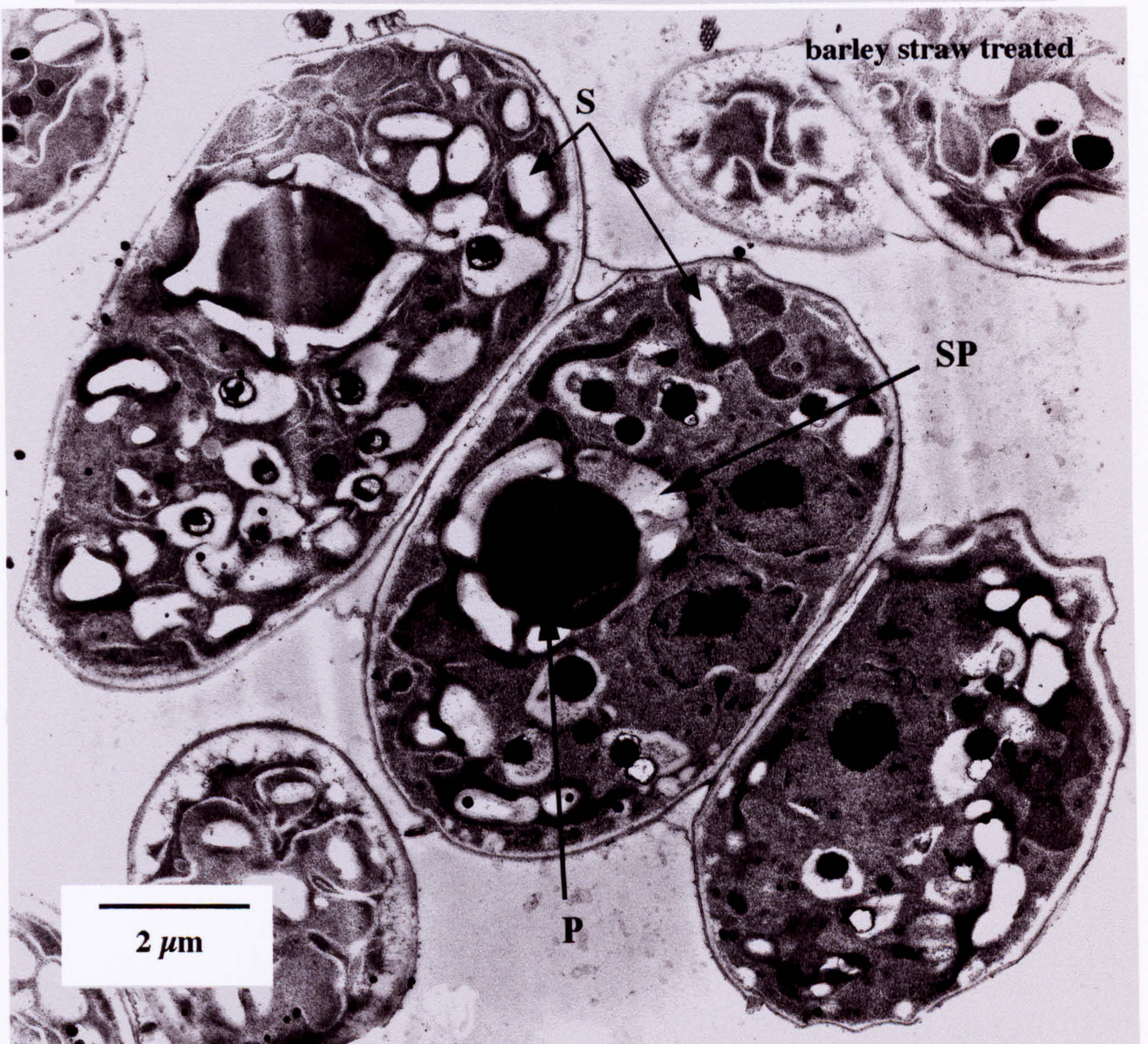
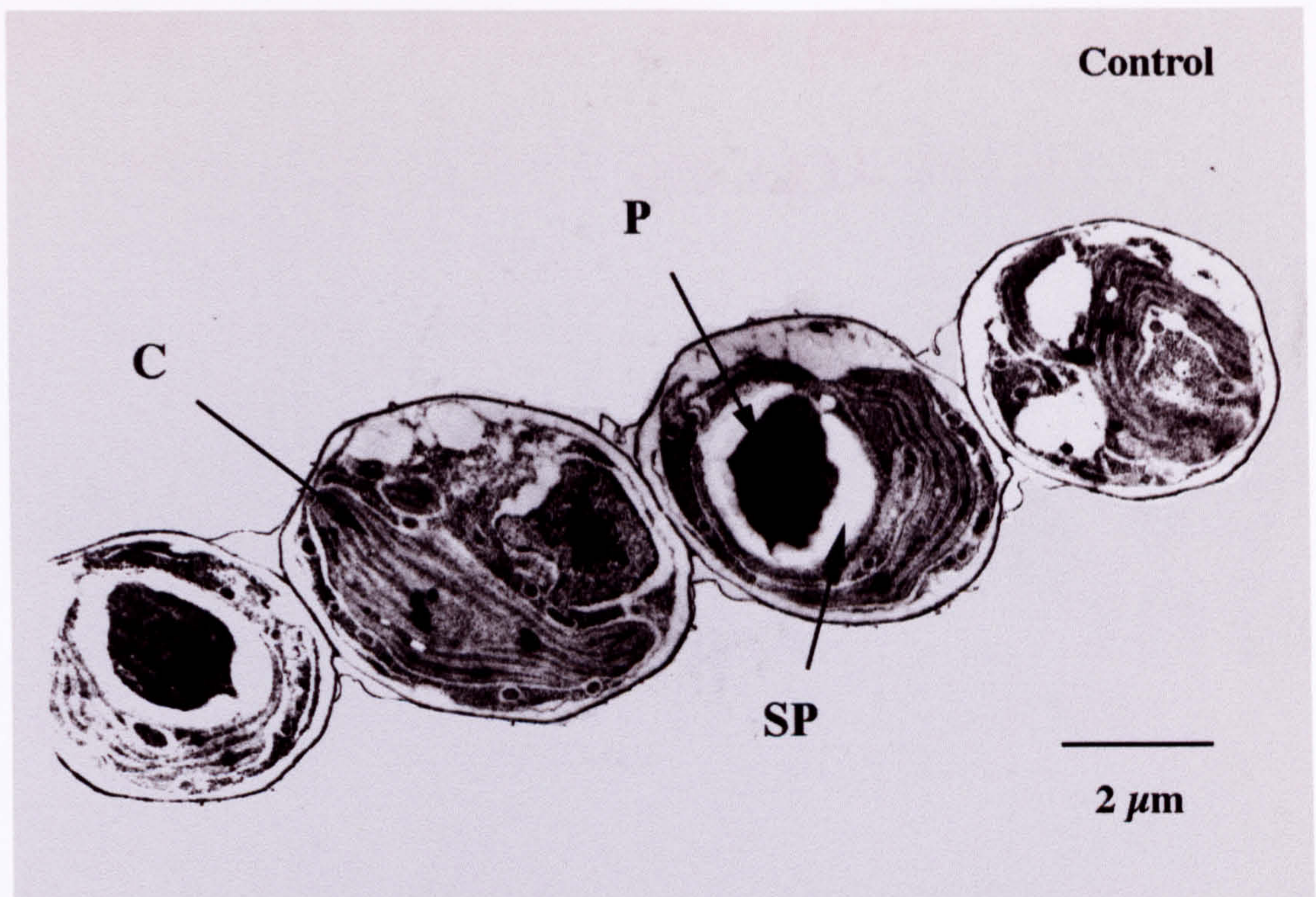


Plate 4.5 Electron micrographs of *Scenedesmus quadricauda* with and without treatment with barley straw after four days growth. C=chloroplast, P=pyrenoid, SP=starch plate surrounding the pyrenoid, S=starch grain (electron translucent area).

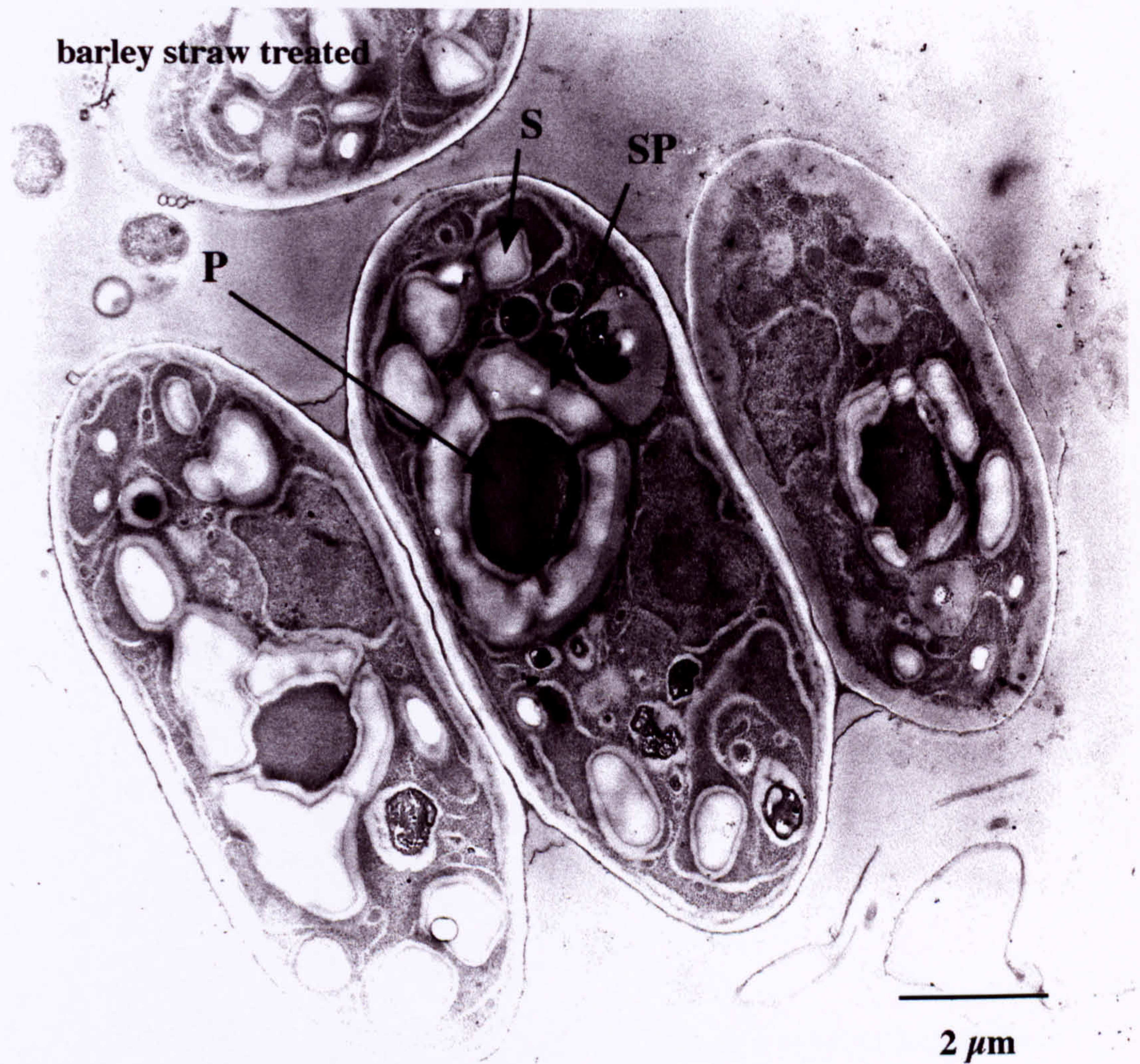
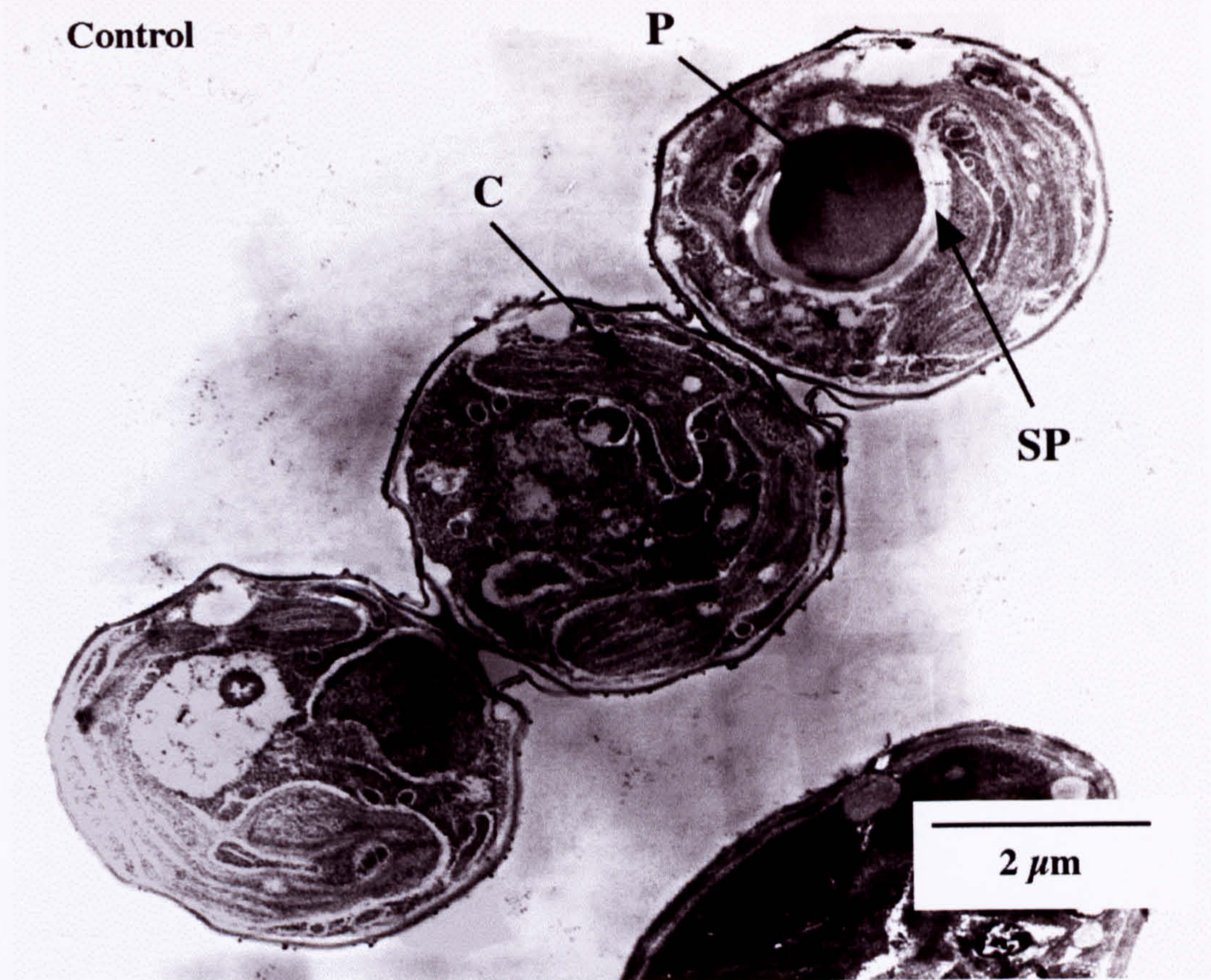


Plate 4.6 Electron micrograph of *Scenedesmus quadricauda* with and without treatment with barley straw after eleven days growth. C=chloroplast, P=pyrenoid, SP=starch plate surrounding the pyrenoid, S=starch grain (electron translucent area).

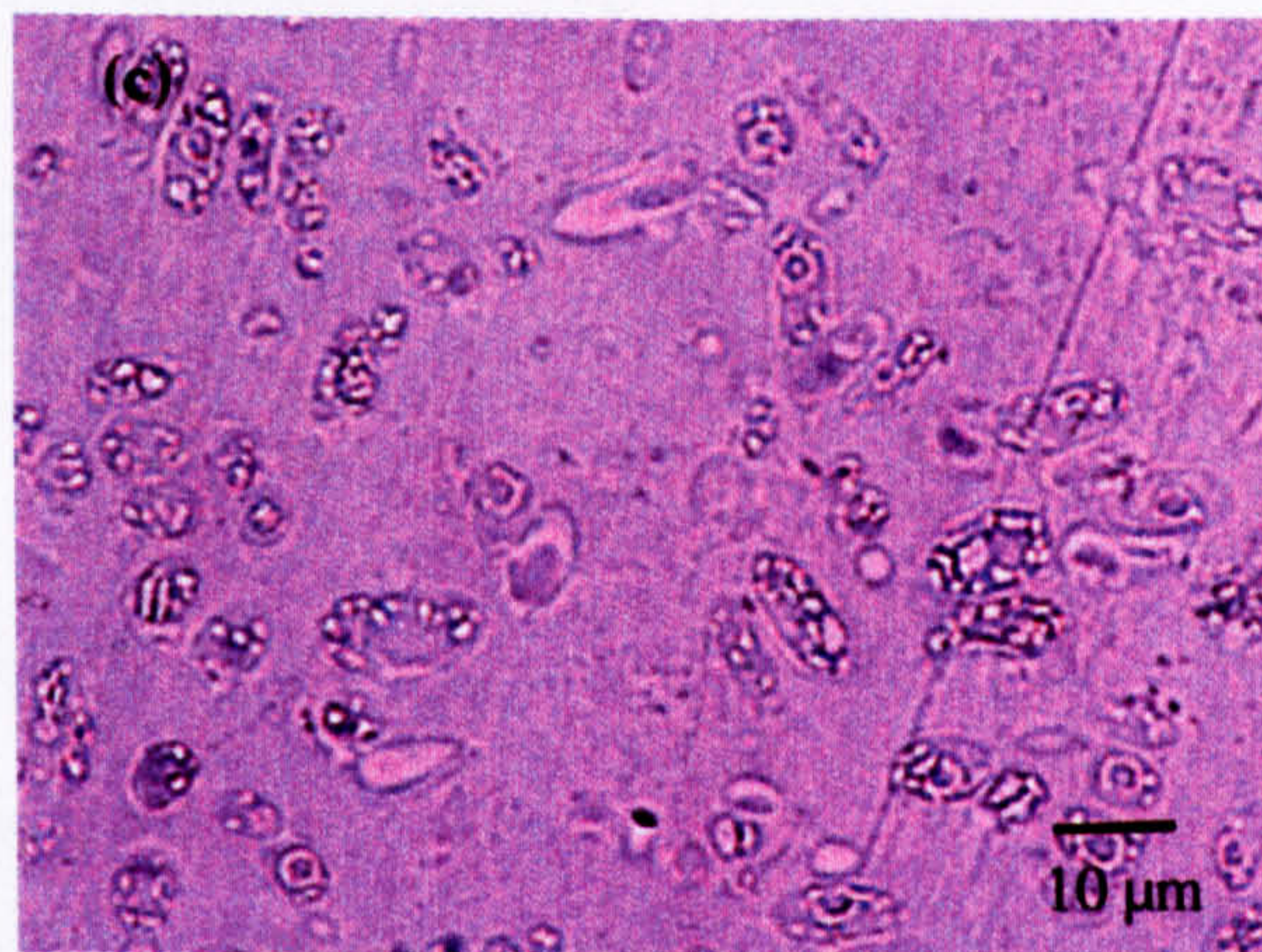
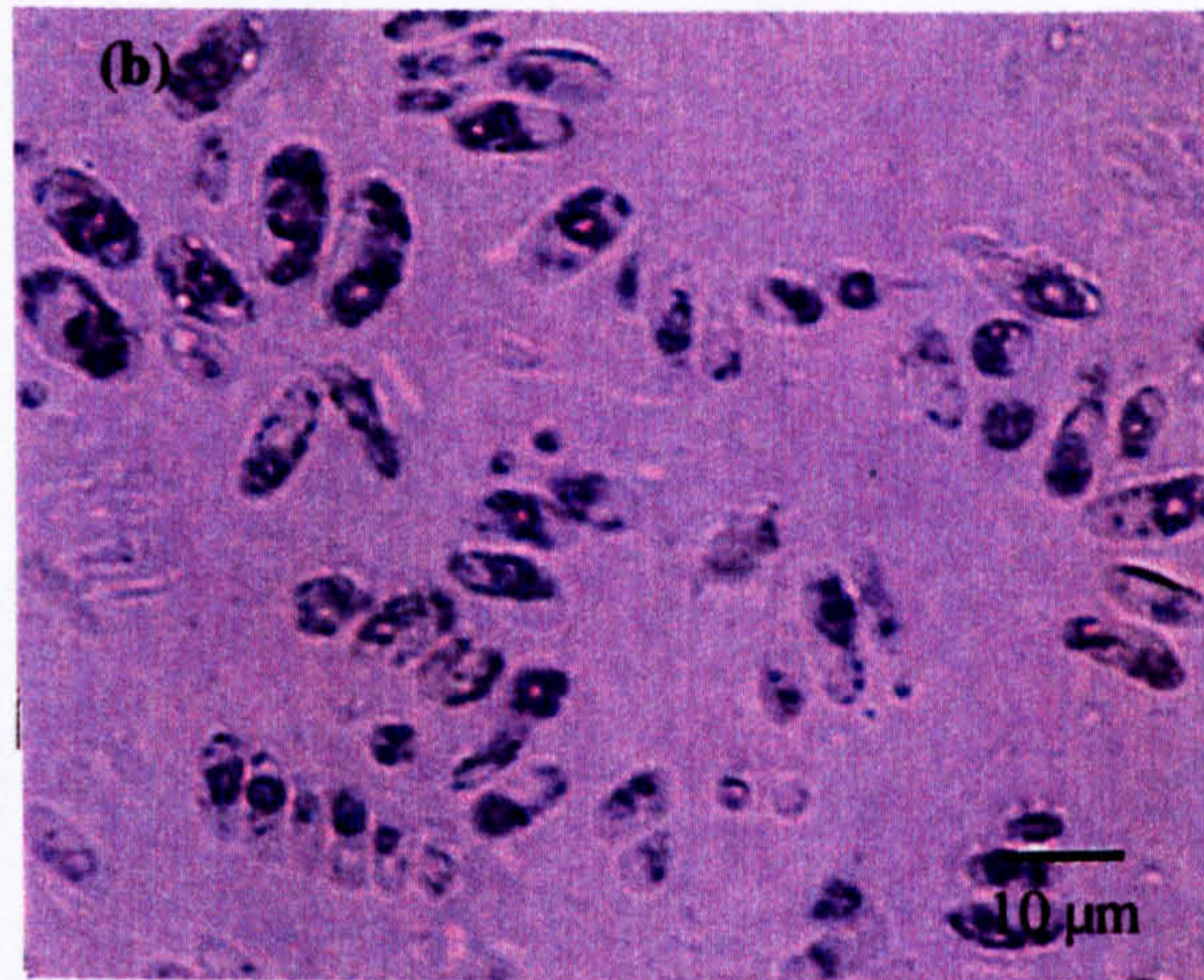
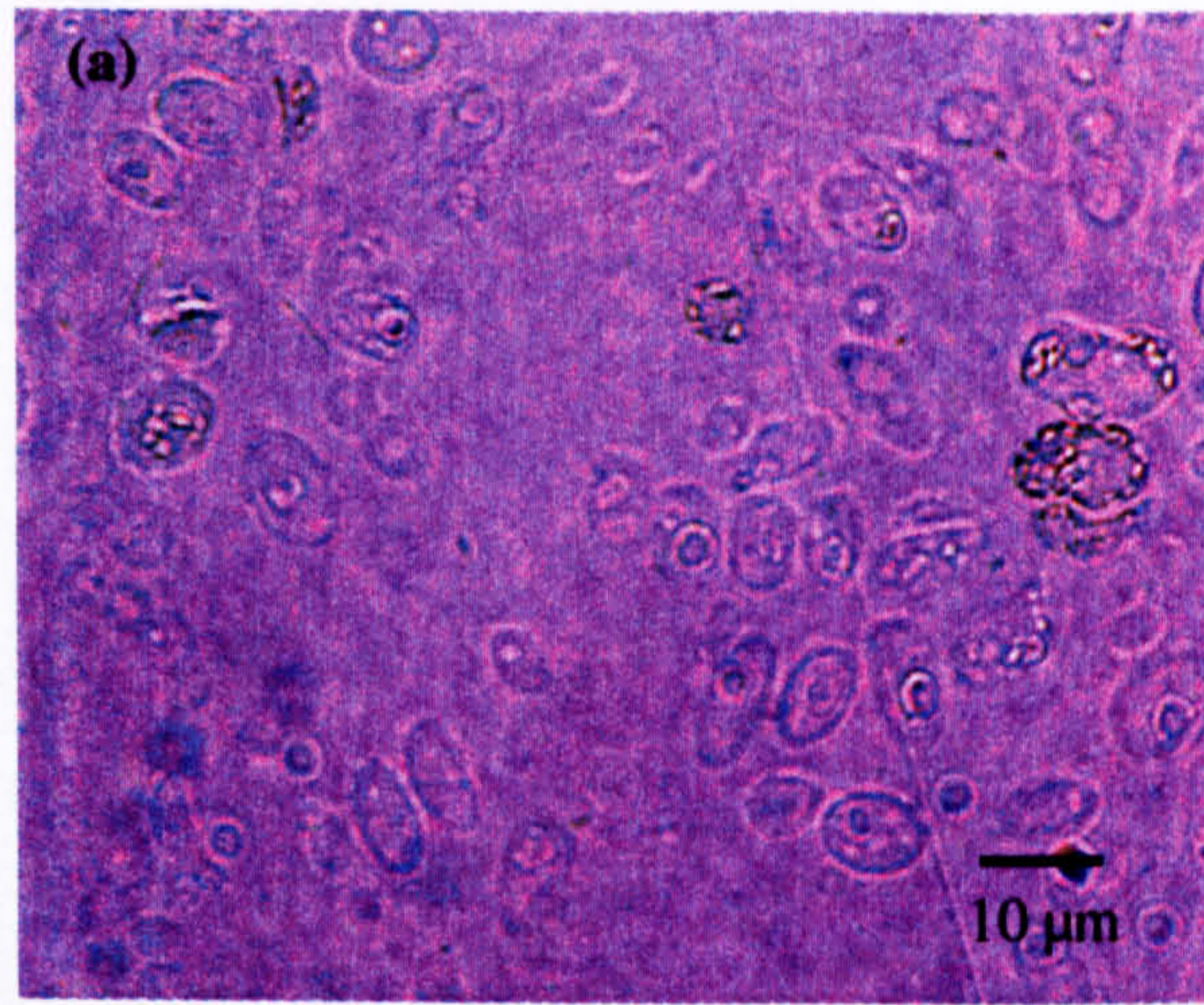


Plate 4.7 Sections of *Scenedesmus quadricauda* treated with iodine and amylase to test for starch. (a) control cells+iodine (b) barley straw treated+iodine (c) barley straw treated+iodine after incubation with amylase. Note blue-black inclusions in (b).

4.4.3 Discussion

For both the *Anabaena* species the thylakoids appeared to remain intact, suggesting that barley straw has no direct effect on these structures whether the cells are inhibited or stimulated. However, replicated experiments using a higher magnification and better quality images are required to verify these findings. The cell walls of *A. flos-aquae* appeared to show signs that the cells were dehydrated. The disruption of the cell wall could be caused by the direct action of barley straw, or conversely, the straw may have produced changes in the cell which became apparent only after the cells were dehydrated in acetone (in preparation for embedding). The fragmentation of the filaments of *A. flos-aquae* was likely to have resulted from the overall growth inhibition, rather than being a phenomenon unique to barley straw inhibition (see section 4.3).

The growth of *Scenedesmus quadricauda* was stimulated in the presence of barley straw but the cells contained one quarter to one third less chlorophyll *a*. The stimulated cells also accumulated starch, both in the four day old cultures and the eleven day old cultures, although cell numbers were stimulated by roughly the same amount in each case. A reduction in cell size could have caused the apparent increase in biomass since biomass was estimated by cell counting. However, the estimation of the biomass of treated cultures appears to be accurate compared to control cultures since the volume of *S. quadricauda* cells was either unchanged or it was slightly increased in the presence of barley straw (see section 3.3.2). Starch accumulation is the opposite to what would be expected if chlorophyll *a* is reduced, since starch is a storage product created from the excess energy and carbon which is not required for cell stasis or growth. Normally, with a reduction of chlorophyll *a* one would expect there to be a reduction in the storage products within the cell, rather than the accumulation of starch and stimulation of growth which was observed with *S. quadricauda*.

Wong *et al.* (1994) found the same type of starch accumulation in the chlorococcalean algae *Chlorella fusca* when grown in the presence of organic effluent

(containing high levels of chlorophenols) from a paper processing factory. In this particular case the accumulation of starch was found to be characteristic of organic toxins and was not found in cells grown in water contaminated with various heavy metals. The cells of *C. fusca* also showed deterioration of other cell organelles, including the chloroplast. However, the results were obtained from cells of *C. fusca* whose growth had been inhibited overall. Wong *et al.* (1994) suggested that the accumulation of starch may have been brought about by the differential disruption of cell organelles. They suggested that the more rapid breakdown of mitochondria compared to the pyrenoids may have resulted in a build up of starch grains. The pyrenoid is a production site for starch; without the mitochondria to carry out respiratory activity, starch is accumulated as an energy reserve. In *S. quadricauda* the pyrenoids appear to be unharmed in straw-treated cells (see Plates 4.5 & 4.6). However, *S. quadricauda* is not growth-inhibited so why and how cell growth and starch accumulation can be stimulated is not known.

The only other possible explanation for the accumulation of starch and stimulation of growth is the assimilation of organic molecules released from the straw (see section 3.7.4.1). *S. quadricauda* has been shown to be able to grow heterotrophically (e.g. Pollinger & Berman, 1976; Soeder & Hegewald, 1988), although whether the decomposition products released from barley straw can be utilized by *S. quadricauda*, or indeed any alga with heterotrophic ability, is not known. Clearly more detailed studies are required to try and obtain a better understanding of the growth characteristics of *S. quadricauda* in the presence of decomposed plant litter.

Further ultrastructural studies on other susceptible species of algae would be useful to determine whether the apparent lack of disruption of the thylakoids observed in *A. flos-aquae* is unique. Greater disruption of cells may occur in eukaryotic algae, although the accumulation of starch and decrease in chlorophyll *a* levels seen in *S. quadricauda* is unique amongst the species tested in this thesis. Previously, copper sulphate, which is known to disrupt thylakoid membranes (Dodge, 1973; Fernandes & Henriques, 1991), was

used as a general growth inhibitor; thus, a suite of toxic substances may have to be used to verify whether ultrastructural changes are unique to plant litter-derived inhibitors.

4.5 Interaction with nutrients

Although it had been shown that barley straw does not cause inhibition via the sequestration of major nutrients (Welch *et al.*, 1990; Gibson *et al.*, 1990), the possibility of an interaction between nutrients and the release of inhibitors from straw had not been investigated. Since the proposed anti-algal compound was thought to be some form of oxidized polyphenol (see section 1.3.1.1), there was a possibility of redox interactions with the transition metals present in the growth medium, mediated by their capacity to exist in many different oxidation states (Sharpe, 1986). Tests were carried out with various nutrient manipulations using the cyanobacterium *Microcystis aeruginosa* since it was known to be highly susceptible to plant litter-derived inhibitors (section 3.6.6).

Of the micronutrients present in JM1 growth medium (see Table 2.1), iron seemed a likely candidate to mediate organic redox reactions since it has two oxidation states of comparable stability and is capable of forming complexes with a wide range of co-ordination numbers and geometries (Sharpe, 1986). Different amounts of iron, as well as manganese and molybdenum, were tested in assays to see what effect they had on the inhibitory potential of plant litter.

4.5.1 Results

Table 4.1 shows that the inhibitory properties of straw remain unaffected with various micronutrient manipulations. *Microcystis aeruginosa* was inhibited whether it was grown with three times the normal micronutrient concentration or whether it was grown without the micronutrient in question. Control cultures did grow in the medium without the

Treatment	Growth (% control)
No micronutrients	1
No EDTA FeNa	1
X3 EDTA FeNa	2
No MnCl ₂ .4H ₂ O	2
X3 MnCl ₂ .4H ₂ O	2
No (NH ₄) ₆ Mo ₇ O ₂₄ .4H ₂ O	1
X3 (NH ₄) ₆ Mo ₇ O ₂₄ .4H ₂ O	2

Table 4.1 Growth of *Microcystis aeruginosa* CCAP 1450/6 with barley straw (800 g m⁻³ dry mass) under different nutrient regimes. *M. aeruginosa* was grown in JM1 medium with changes in nutrients as shown in the table. Micronutrients denotes stock number six from Table 2.1. Inhibition of growth was significant (p <0.001) in all cases when compared to control cultures with the same nutrient manipulation.

addition of the various micronutrients but they began to show signs of senescence earlier than would be expected if using standard JM1 medium. The inhibition of *M. aeruginosa* was not an artefact produced by the microflora of the straw sequestering trace quantities of micronutrients left in the flasks, since the addition of extra micronutrients to the already inhibited cultures still produced no growth in the presence of barley straw (results not shown). The initial incubation and decomposition of the straw (see section 2.2.1) under different nutrient regimes such as distilled water (no nutrients), JM1 growth medium and JM1 growth medium without micronutrients still produced inhibition of *M. aeruginosa* (results not shown).

4.5.2 Discussion

The growth of the controls suggests that the initial inoculum was able to grow using internal reserves of nutrients which were already present in the cells; these were eventually exhausted and the culture started to senesce earlier than would otherwise be expected. The inhibitory potential of the straw when incubated under various nutrient regimes (including distilled water) remained unchanged, showing that the incubation period of the straw (section 2.2.1) required no excess nutrients other than those already present in the straw. This provides evidence that the nutrients already present in the straw may be enough to allow its inhibitory potential to be realized, thus hindering the present work in trying to eliminate various micronutrients from the growth medium. The addition of triple the concentration of metal resulted in the same inhibitory effect as elimination of the metal. Any possible interaction of the transition metals with the plant litter-derived inhibitor(s) must, therefore, be via very small concentrations of metals which are already incorporated into the barley straw. For practical purposes in the field, any interaction with those micronutrients tested here can be discounted since the straw will provide enough of its own. However, the possibility exists that other or higher concentrations of nutrients may play a role in the production of inhibitory compound(s) (see section 5.7.3).

The possibility exists that the inhibitor(s) are affected in a manner that is not shown in these experiments. The inhibitory potential could have been reduced or increased under the different nutrient regimes but because *M. aeruginosa* was the most susceptible species tested, it would still show strong inhibition. Further experiments on a less susceptible species would, perhaps, provide further evidence as to the interaction with micronutrients.

4.6 Conclusions

When treated with decomposed plant litter, very few species of algae showed any changes in their morphology/structure that could be observed at the level of light microscopy. Of those species that did show changes in morphology/structure, the changes were found to

result from a general inhibition of growth rather than them being a specific effect of the litter-derived inhibitors. Ultrastructurally there appears to be no disruption of the photosynthetic membranes of *Anabaena flos-aquae* when inhibited by barley straw. Further detailed studies using both eukaryotic algae and other species of cyanobacteria are required to see if this phenomenon is widespread. However, it seems most unlikely that any ultrastructural changes could be attributed specifically to plant litter since it is difficult to distinguish between cause and effect.

The fact that cell numbers are reduced in susceptible species shows that cell division is hindered. No major size reduction was seen in the main body of the cell in *S. pingue* and cell expansion was not inhibited in *P. boryanum* (section 4.2.2) or seen to be inhibited in any of the other species which were assayed. The reduction in prong length observed in *Staurastrum pingue* and *Pediastrum boryanum* suggests that cytoskeletal functions may be disrupted with both litter-derived inhibitor(s) and copper sulphate.

The growth of *Scenedesmus quadricauda* in the presence of barley straw is very unusual. Further studies are required to resolve the apparent paradox in the growth characteristics of *S. quadricauda*, whereby its growth appears to be unaffected or stimulated in the presence of barley straw, yet chlorophyll *a* levels are reduced and the cells appear to accumulate starch.

Manipulation of the nutrients used in the JM1 growth medium does not appear to affect the inhibitory potential of the straw and any possible interaction can probably be brought about by the amounts already incorporated into the straw.

Chapter 5

The relationship between algae and macrophytes in barley straw-treated drainage channels

5.1 Introduction

Shallow waterbodies of up to 3-4 metres deep are often dominated by macrophytes (which does not include filamentous algae for the purposes of this chapter), since insufficient light penetrates for germination and growth in deeper waters. The presence of macrophytes is often regarded as being beneficial since they increase habitat diversity and are seen to have greater amenity value than waterbodies dominated by algae. Large growths of macrophytes can become a nuisance in boating lakes and drainage channels (Westlake, 1975), but they are generally preferred over nuisance growths of algae since macrophytes are easier to manage. Eutrophication of a waterbody can disrupt the dominance of macrophytes and may produce a switch in the ecosystem to one which is dominated by algae (Irvine *et al.*, 1989; Scheffer *et al.*, 1993).

5.1.1 Macrophyte domination

The significant advantage that macrophytes have over algae is their access to the sediments. Most macrophytes are rooted in the sediment, which tends to be richer in nutrients than the overlying water (Moss *et al.*, 1996a). Just below the sediment surface, sediments become anaerobic due to the action of decomposer organisms and the lack of oxygen diffusion from the upper layers of sediment. The anaerobic environment helps to keep phosphate in solution, and therefore available to plant roots, whereas phosphorus levels may be much lower in the overlying water column (Marsden, 1989; Moss *et al.*, 1996a). The surface layer of sediment is more aerated; here, bacterial activity can produce ammonium as a decomposition product, which is immediately taken up by macrophytes or

converted to nitrate in the sediment. Nitrate is either taken up by macrophytes or is usually lost to the system via microbial denitrification in anaerobic microsites (Moss, 1987). In this way most of the nitrogenous compounds are either immediately taken up by the macrophytes or removed by denitrification, thus depriving the algal communities of nutrients. Macrophytes are more competitive than algae under these conditions because, as well as being able to take up nutrients from the water column, they are also able to utilize and recycle nutrients which are present in the sediment. The competitive superiority of macrophytes in pristine waters allows them to sequester nutrients and shade out most of the algal growth that would otherwise take place.

A number of other factors can contribute to macrophyte dominance, such as the production of algal growth inhibitors (Phillips *et al.*, 1978; Scheffer *et al.*, 1993). For instance, *Myriophyllum spicatum* L. is known to produce hydrolysable polyphenols that can inactivate extracellular enzymes of algae, producing algicidal effects (Gross *et al.*, 1996). A more indirect mechanism that promotes macrophyte dominance is the utilization of stands of macrophytes as refuges for invertebrate grazers of phytoplankton, particularly cladocerans such as *Daphnia* spp. (Beklioglu & Moss, 1996; Moss *et al.*, 1998). During the day *Daphnia* can seek refuge in stands of macrophytes, where the risk of predation by small fish is lower, whereas at night the *Daphnia* can disperse more freely to graze phytoplankton when the fish cannot see them. In this way the presence of macrophytes helps to exert a selection pressure against the growth of algae by providing habitat for algal grazers.

5.1.2 Eutrophication

Shallow lakes are prone to eutrophication since they tend to occur in low-lying areas surrounded by agricultural or urban land. The increase in nutrients can have a detrimental effect on the stable state of macrophyte domination.

Initially, as nutrient levels increase, the biomass of macrophytes may begin to increase, but the diversity of the plant community starts to decrease (Moss *et al.*, 1996a). Competitive species such as *Potamogeton pectinatus* and *Ceratophyllum demersum* will become dominant, but at higher nutrient levels the plant community becomes less stable and is liable to switch to an alternative stable state characterized by algal dominance. The increased nutrient levels in the water column allow epiphytic and filamentous algae to grow on the macrophytes. Epiphytic growth reduces the growth of macrophytes through shading, which in turn allows greater light penetration into the water column and produces more favourable conditions for algal growth (Phillips *et al.*, 1978; Moss *et al.*, 1996a). The reduction of macrophyte growth produces a concomitant reduction in the secretion of phytoplankton growth suppressants, increasing further the competitiveness of algae throughout the whole water column.

5.1.3 Algal domination

Once an aquatic system has switched to a state of algal domination there are many processes in place which operate to prevent the system from returning to macrophyte domination, even if nutrient levels are reduced. Algal dominance is promoted by an earlier start to growth in the year and the ability of algae to attain a large biomass quickly, since energy does not have to be partitioned into root and rhizome tissue. The decrease in macrophytes often means that the water becomes more turbid, since there are no plant roots to bind the sediments (Moss, 1987; Blindow *et al.*, 1993). Turbid water prevents light penetration to the young shoots of macrophytes and this shading effect is exacerbated by the growth of algae in the overlying water column.

The elimination of macrophytes removes the refuges for invertebrates, allowing the heavy grazing of invertebrates by predator fish. High pH and reduced average oxygen levels in algal dominated lakes may disfavour piscivorous fish, allowing greater numbers of zooplanktivorous species (Moss *et al.*, 1996a). The increased grazing on zooplankton means the grazing pressure on phytoplankton is reduced, adding further to the increase in

algal biomass. As well as reducing light levels, algae may influence the physical and chemical properties of water in ways that promote competitive exclusion of macrophytes (Cate *et al.*, 1991). For instance Simpson & Eaton (1986) have shown that the filamentous algae *Cladophora glomerata* and a *Spirogyra* sp. can out-compete the macrophyte *Elodea canadensis* under conditions of high pH and low carbon dioxide concentration; this effect is probably due to the stronger induction of carbonic anhydrase and more effective use of HCO_3^- in the two algal species.

5.1.4 Alternative states

The predominant view is that a shallow water body will be in one of two alternative states: it is either dominated by macrophytes, or dominated by phytoplankton and/or filamentous algae (Scheffer *et al.*, 1993; Blindow *et al.*, 1993; Stephen *et al.*, 1998). Each state has its own mechanisms by which it preserves dominance over a wide range of nutrient concentrations, although the higher the nutrient levels the greater the risk of the system switching from macrophyte to algal dominance (Scheffer *et al.*, 1993; Moss *et al.*, 1996a). An increase in nutrients alone cannot displace aquatic macrophytes altogether (Moss, 1987; Moss *et al.*, 1996a); additional mechanisms are required, with the switch often being caused by management techniques. The removal of excessive macrophyte growth by mechanical or chemical means can exacerbate problems, since it may allow algae to grow excessively where once only macrophytes grew. Various forms of 'pollution' in a water body, such as pesticides or an increase in salinity, can also reduce phytoplanktivorous feeders (Moss, 1987). However, Cate *et al.* (1991) suggested that in some cases a mixture of these states may occur, since in some Dutch drainage ditches filamentous algae and macrophytes can co-occur if the nutrient levels are not excessively high.

5.2 Reversing the switch

As mentioned previously, once an aquatic community has become dominated by algae various mechanisms prevent the system from being easily switched back to macrophyte

domination, even if the nutrient levels are reduced. Attempts have been made by various workers to induce the switch back to macrophyte domination, normally using a combination of biological and physical management techniques.

The removal or prevention of nutrient inputs is commonly undertaken (see section 1.3) to provide the foundation for a reduction of excessive algal growths, but this technique alone rarely works to reduce algal growth (Marsden, 1989; Scheffer *et al.*, 1993). Biomanipulation has been widely employed in tandem with reduced nutrient levels to help reduce algal growths (Mason, 1991; Moss *et al.*, 1998; Stephen *et al.*, 1998). For example, the addition of piscivorous fish and/or removal of zooplanktivorous fish has been used as a means to reduce grazing pressure on phytoplankton feeders, leading to an increase in grazing pressure on phytoplankton and subsequently a reduction in algal biomass. Removal of bottom-feeding fish such as carp (*Cyprinus carpio* L.) and bream (*Abramis brama* L.) reduces the turbidity of the water, producing conditions more favourable for the regrowth of macrophytes (Scheffer *et al.*, 1993; Moss *et al.*, 1996a). Macrophytes can also be manually introduced into a water body to help with recolonization where natural inocula are absent because, for example, they are being destroyed by birds or where the sediment conditions are inhibiting regrowth (Moss, 1987; Weisner, 1997).

Artificial reduction of the water level may also play a part in the switch back to macrophyte dominance, although this has yet to be used for lake restoration (Scheffer *et al.*, 1993). Blindow *et al.* (1993) found that natural changes in water levels are known to cause switches in two Swedish lakes, possibly via changes in light availability or via catastrophic events such as drying out.

5.3 Field trials

In recent years, drainage engineers for the Middle Levels Commissioners based in Cambridgeshire, U.K. have started trials using barley straw to control growths of algae in land-drainage channels. The major nuisance algae is *Vaucheria dichotoma* (L.) Martius

(Xanthophyceae, Heterokontophyta) which can grow attached to the bottom sediments causing blockages in the channels. One of the problems associated with *V. dichotoma* was that it appeared to have become resistant in some channels to the herbicide terbutryn (Gibson & Barrett, 1989), which was used as the normal method of control. Biomanipulation of the channels and removal of zooplanktivorous fish was not an option since the channels could not be blocked off from other channels connecting to them. Also, the algal nuisance was caused by filamentous algae, so there was little point in trying to increase phytoplankton grazers. However, an increase in macrophytes would be advantageous and easier to manage than algae (G. Cave, pers. comm.). Previous studies using barley straw in the field had only qualitatively assessed the status of macrophytes (Welch *et al.*, 1990; Everall & Lees, 1996). To my knowledge no-one had ever quantitatively assessed algal and macrophyte biomass to see whether a switch to macrophyte domination could be achieved, although a switch to macrophyte domination had been reported by Caffrey & Monahan (1999) and Barrett (1994). Field trials were therefore started using barley straw as a control method to determine, firstly, if it could control algae in the drainage channels and, secondly, whether a switch to macrophyte domination could be induced. The opportunity arose to carry out surveys of two sites where barley straw was being used and to assess the relative dominance of macrophytes and algae in the drainage channels.

5.4 Description of the sites and straw application

The sites which I was able to survey were chosen largely for convenience to the Middle Levels Commissioners, since one site was on private land. Both sites were situated in an arable farming area adjacent to fields of various crops. At both the sites water levels varied between 1-2 metres and there were large growths of filamentous algae but very little phytoplankton.

5.4.1 Monks Lode

This channel is situated near the village of Conington in Cambridgeshire, U.K. (national grid reference TL212858). The channel forms part of a long-interconnecting series of channels in the region, but only part of the channel was used in the field trial. The channel suffered mainly from nuisance growths of *Vaucheria dichotoma* attached to the bottom sediments but often had floating mats of *Cladophora glomerata*, *Oedogonium* spp. and *Spirogyra* spp. A 2-3 metre wide embankment separated the channel on both sides from the surrounding fields.

Whole bales of straw were placed in the BB site in 1994 by drainage engineers (see Figure 5.1) at a dose of ca. 70 g m⁻³. More straw was placed in the BB site in 1995 but the old straw was not removed. In both cases whole bales of straw were used and not supported by any means of flotation; therefore, it was assumed that the centre of the bales would rot anaerobically and not contribute to the production of algal inhibitor(s) (P.R.F. Barrett, pers. comm.). In early May 1996, straw was introduced to both the BB and AB sites attached to polyurethane floats anchored in the centre of the channel, at a dose of approximately 17 g m⁻³. This prevented the straw from sinking and allowed it to decompose at the water surface, in the presence of oxygen. Laboratory tests with the polyurethane foam used in the straw floats showed that it was not inhibitory to *Microcystis aeruginosa*, the most susceptible species assayed in Chapter 3 (results not shown).

In early 1997 all of the polyurethane floats and straw were accidentally removed from both the AB and BB sites during routine maintenance, although the remnants of two bales of straw were still present in the BB site from the 1995 application.

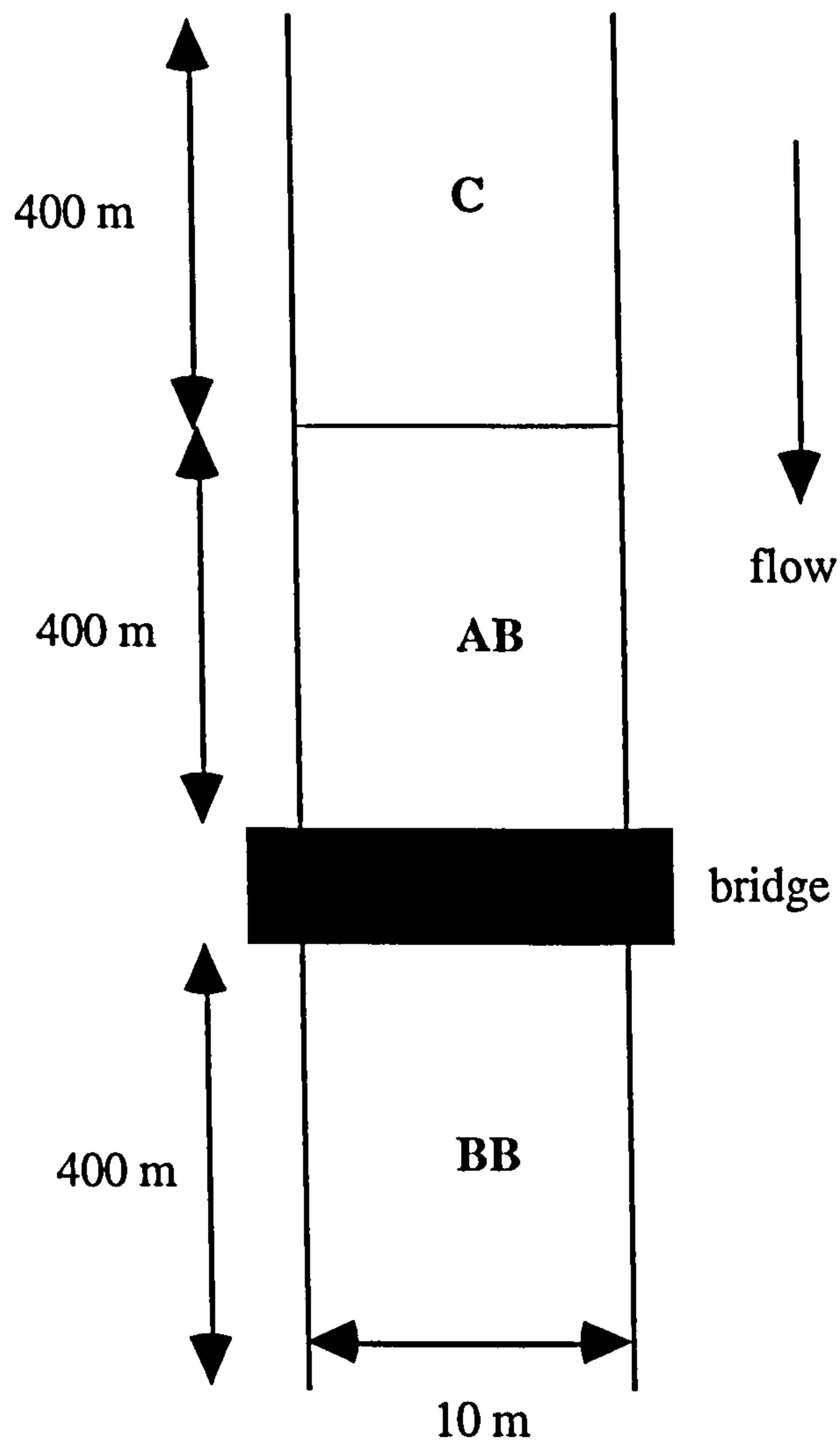


Figure 5.1 Plan of the Monks Lode experimental site. C = control site, AB = above bridge site where straw was first applied in 1996, BB = below bridge site where straw was first applied in 1994 (see text for details). Pumping introduced a unidirectional flow of water into the channel periodically.

5.4.2 Binnimoor drain

This channel is situated near the Cambridgeshire town of March, U.K. (national grid reference TL436945) and has a history of algal nuisance in the form of *Vaucheria dichotoma* attached to the bottom sediments. It also often has growths of *Enteromorpha flexuosa* (Wulfen) Agardh floating on the water's surface along with smaller amounts of *Oedogonium* spp., *Spirogyra* spp. and *Cladophora glomerata*.

The experimental area was divided into four segments (see Figure 5.2), which had all been cleaned out mechanically in the winter of 1996/97. Straw was first applied in late

April 1997 using the same type of polyurethane floats that were used in Monks Lode in 1996. Straw was applied to the S1 and S2 sections at a dose of approximately 20 g m⁻³ spaced out evenly along the two sections in 19 straw floats. In April 1998, additional floats and fresh straw were added to both the 'S' sections to give a dose of approximately 30 g m⁻³. A floating boom was also added at the C1 site to prevent floating algae (mainly *Enteromorpha flexuosa*) from getting into the S1 site.

The set up of the site was not ideal for the experiment (but was unavoidable since it was originally set up by the Middle levels Commission) since slow water flow, introduced by periodic pumping, could flow into the C2 section. It was considered necessary to survey the C2 site as a control site because the sites upstream and downstream of the culvert appeared visually to be different. The S1 and C1 sites were smaller ditches (see Figure 5.2) and appeared to contain more floating algae than the sites downstream. These floating algae were prevented from floating downstream into the other two sites by the culvert. Any algal inhibition would be greatly diminished over the entire length of the C2 site due to the short-lived nature of the inhibitory compound(s) (Welch *et al.*, 1990). By comparing surveys carried out at the extreme downstream end of the C2 site to those carried out in the S2 site, any algal inhibition that did occur could be detected.

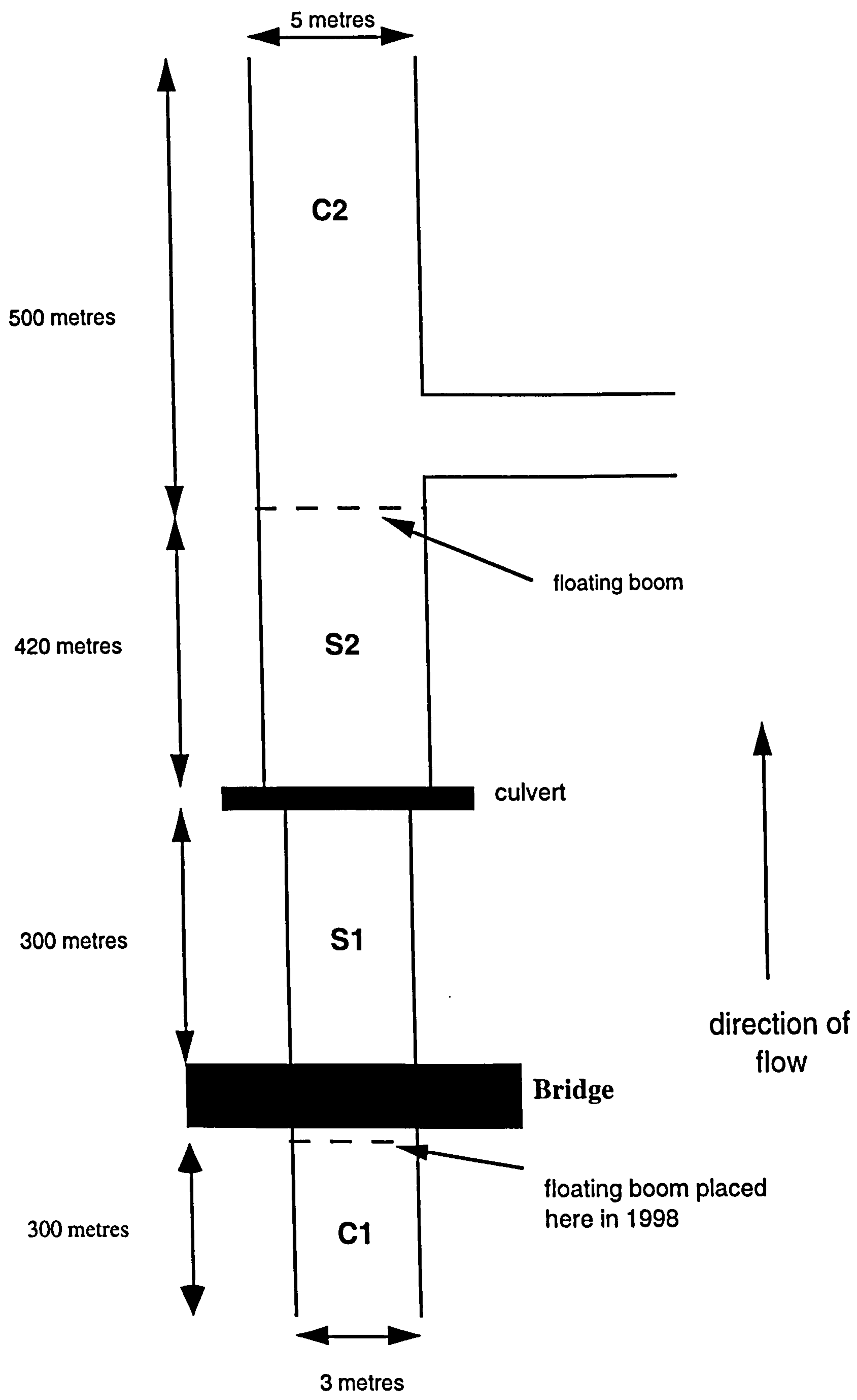


Figure 5.2 Plan of the Binnimoor experimental site. S = straw applied, C = control sites (see text for further details). Pumping introduced unidirectional flow into the channel periodically.

5.5 Methods

Surveying was based upon a point-survey system since the collection of macrophytes and algae for biomass analysis was problematical and too time-consuming for the size of the channels and resources available. A rope, which had been marked at 30 cm intervals, was placed across the width of the drain. Every 30 cm across the drain a score was given as to the presence (score=1) or absence (score=0) of algae or macrophytes on the surface or below the water's surface; it was also noted whether algae were present underneath the macrophytes or *vice versa* (score=0.5). Four transects were taken, spaced evenly across each treatment in both field sites although, for the Binnimoor site, the C2 treatment was surveyed within a 200 metre stretch at the extreme down stream end of the site (see section 5.4.2). Monks Lode was surveyed in 1996 and 1997 and Binnimoor drain was surveyed in 1997 and 1998. Both field sites were surveyed three times a year in June, July or August and October and the data pooled for each individual year. Percentage cover of both algae and macrophytes in each section of the field sites was calculated from the scores given to each vegetation type compared to the total number of observations. For statistical analysis, the ratio of algae to macrophytes for each section of the field sites was calculated for each individual year of study. Statistical analysis was carried out by means of an analysis of variance on Log (n+1) transformed data (since zero's were present in the data); where significance was found, Tukey tests were performed to determine where the differences were. All analyses were performed in Statistica 5.1 (Statsoft Inc.).

Nitrogen and phosphorus analysis was carried out on the water in both sites in 1998. Sub-surface samples (ca. 20 cm deep) were collected in polypropylene bottles (rinsed three times with sample water) and chemical analysis performed within five hours on GF/C (Whatman) filtered samples. Nitrate was measured using a DR100 colorimeter test kit (Hach) and expressed as NO₃-N, and filterable reactive phosphorus (FRP) was determined using standard methods based on molybdenum blue formation (HMSO, 1992).

5.6 Results

5.6.1 Monks Lode

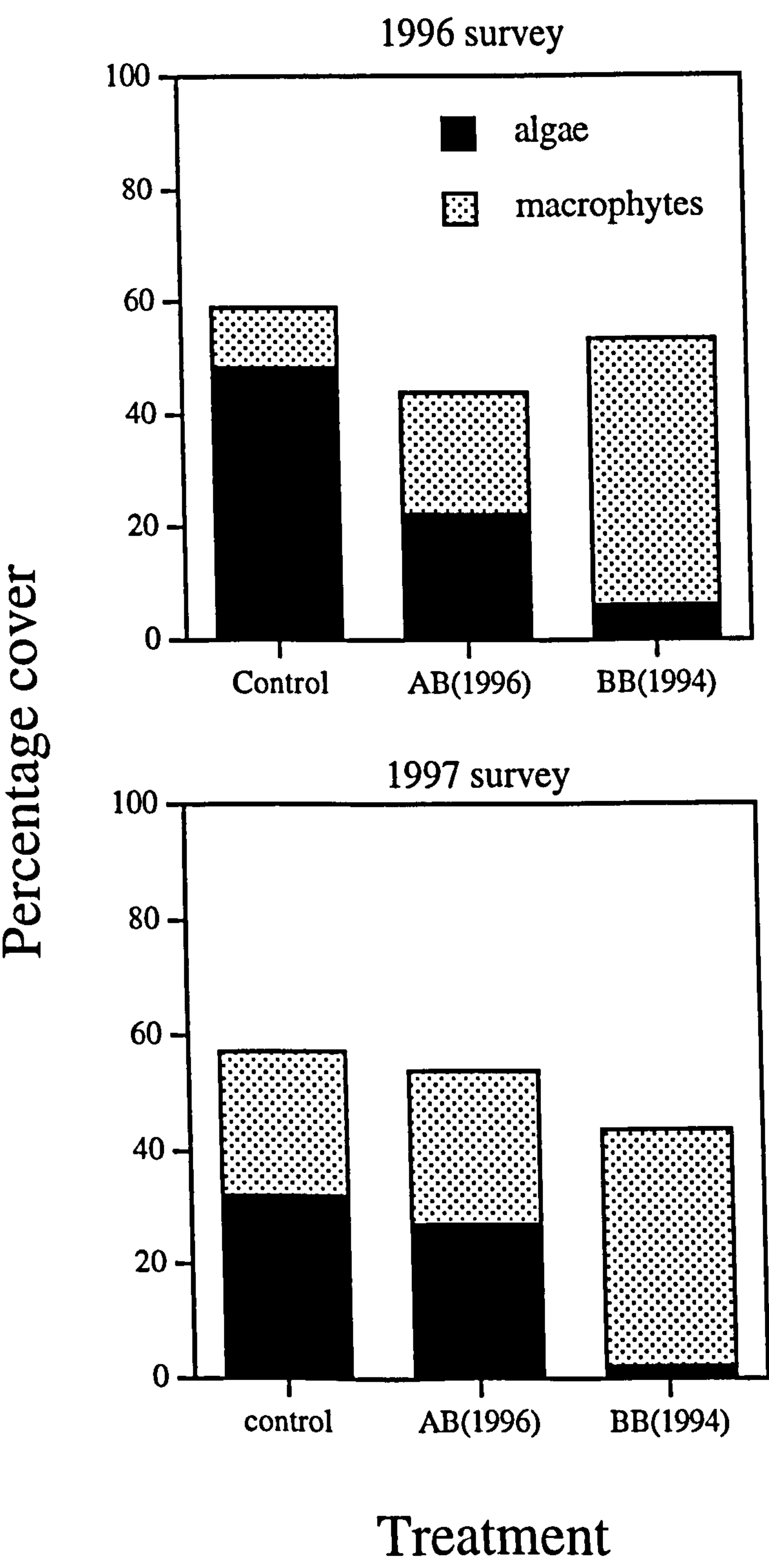


Figure 5.3 Percentage cover of macrophytes and algae in the three different treatment areas of Monks Lode. The years in parentheses denote when barley straw was first applied to the site (straw was removed in 1997). A two-way ANOVA of the ratio between algae and macrophytes on Log (n+1) transformed data shows there to be significant differences between sites ($p < 0.001$) but no significant difference between years ($p > 0.1$).

In 1996 the control section clearly had a significantly higher ratio of algae:macrophytes than the two sections which had been treated with straw (see Figure 5.3). The ratio of algae:macrophytes in the BB section, where straw had been used for two years previous to 1996, appeared to be the lowest, hence, the BB section was dominated by macrophytes. In the AB site, where straw had been applied only in the year of the survey, there appeared to be a 1:1 ratio between algae and macrophytes, although from Table 5.1 this was significantly different from the control site ($p < 0.05$) showing that the straw was having some effect in inhibiting algal growth.

Year	Comparison	Significance
1996	C x AB	*
	C x BB	***
	AB x BB	NS
1997 (straw removed)	C x AB	NS
	C x BB	*
	AB x BB	**

Table 5.1 Summary of comparisons between treatments in different years at Monks Lode. Significance testing was performed by means of Tukey tests on Log (n+1) transformed data of the ratio of algae to macrophytes. NS = Not significantly different at $p = 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

In 1997 only the BB site had a significantly higher proportion of macrophytes ($p < 0.05$), with the AB site showing no difference in the ratio of algae:macrophytes from that of the control. The control site did appear to have a higher proportion of macrophytes in 1997 than in 1996 but this difference was not significant ($p > 0.3$).

Nutrient analysis (Table 5.2) showed that phosphorus levels were not particularly high in 1998. Phosphorus in the BB site in April appeared to be higher than the rest, but

this was probably due to water being pumped from surrounding ditches into this site on the day of sampling, although the amount of nitrate was no higher than in the other two sites.

Date	Site	Nutrient	
		NO ₃ -N (mg l ⁻¹)	FRP (µg l ⁻¹)
April	C	15	5
	AB	10	12
	BB*	13	26
June	C	5	4
	AB	5	4
	BB	6	4

Table 5.2 Nutrient analysis for Monks Lode. * water was being pumped into this section from the drains of surrounding fields on the day of sampling.

5.6.2 Binnimoor drain

A two-way analysis of variance of the surveys in 1997 and 1998 showed there to be a significant interaction between site and year ($p < 0.001$), implying that the treated sections responded differently in different years. Examination of the raw data and the results shown in Figure 5.4 suggested that this interaction was brought about by the differences in the S1 and C1 sections between the two years, since they appeared to have much higher growths of algae in 1997 than in 1998. Further analysis was carried out using a one-way analysis of variance to show differences in the ratio of algae:macrophytes in separate years.

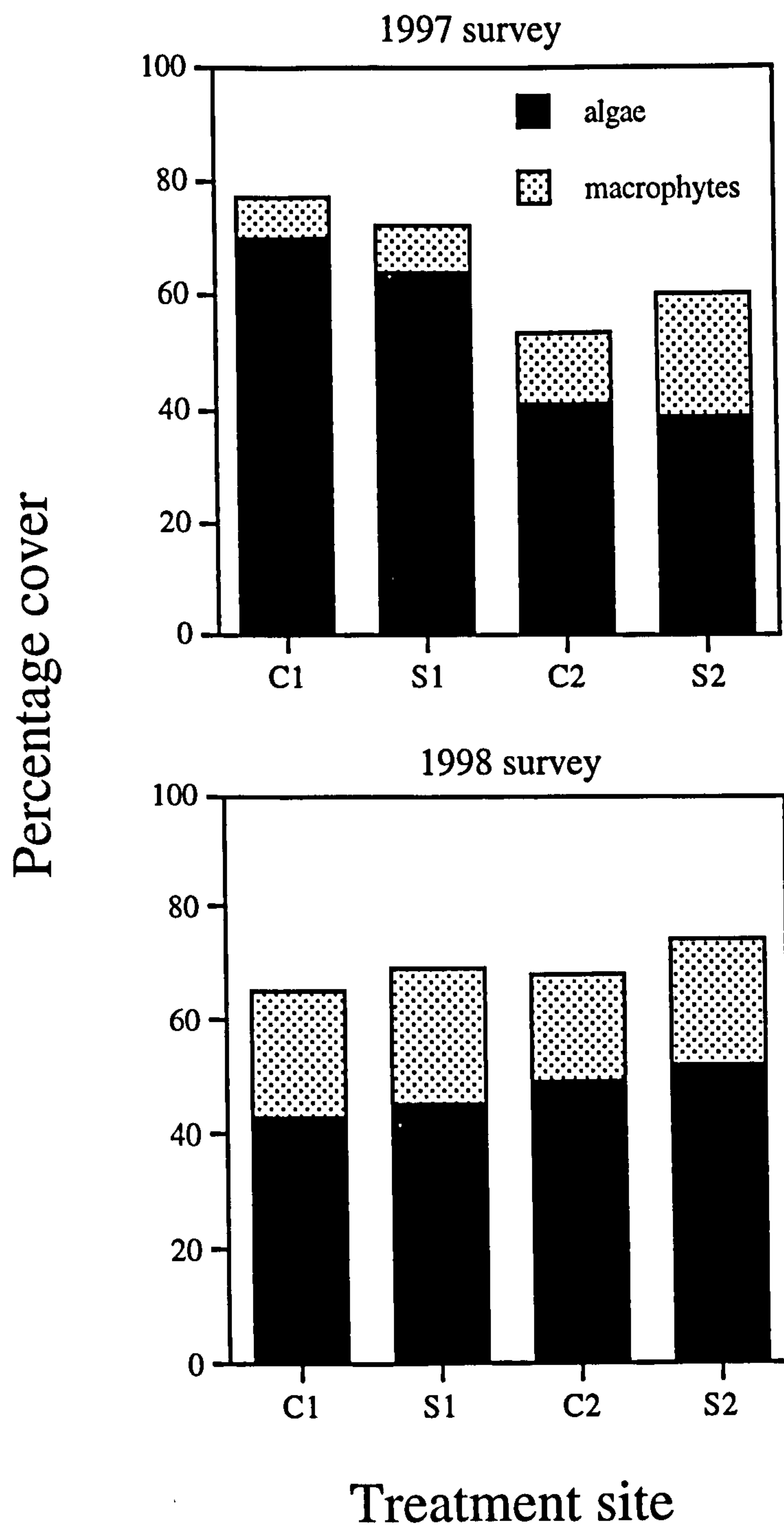


Figure 5.4 Percentage cover of algae and macrophytes in Binnimoor drain in 1997 and 1998.

From Table 5.3, in 1997 the C2 & S2 sites ($p > 0.5$) and the C1 & S1 sites ($p > 0.9$) showed no significant differences in the ratio of algae:macrophytes. The fact that all of the other comparisons were significantly different suggested that both the '1' sites were different from both the '2' sites, therefore comparisons could not be made between '1' and '2' but only **within** '1' and '2'. In each case there appeared to be no significant reduction in

Comparison	Significance
C2 x S2	NS
C2 x S1	*
C2 x C1	*
C1 x S1	NS
C1 x S2	*
S1 x S2	*

Table 5.3 Summary of comparisons in Binnimoor drain in 1997. Significance testing was performed by means of Tukey tests on Log (n+1) transformed data of the ratio of algae to macrophytes. NS = Not significantly different at $p = 0.05$; * $p < 0.05$.

the amount of algal growth compared to macrophyte growth in the sections treated with straw.

A one-way analysis of variance of the 1998 data showed no significant differences between all of the sites ($p > 0.5$), indicating that barley straw was having no effect on reducing the algae:macrophyte ratio (see Figure 5.4).

In Binnimoor drain there appeared to be considerably more nitrate and phosphorus than in Monks Lode (cf. Tables 5.2 & 5.4). Levels of both nutrients decreased as the growth season progressed, with there being no detectable phosphorus in June (apart from a small amount in the C2 site) or August.

Date	Site	Nutrient	
		NO ₃ -N (mg l ⁻¹)	FRP (µg l ⁻¹)
April	C1	45	68
	C2	40	57
	S1	50	18
	S2	35	37
June	C1	16	0
	C2	13	2
	S1	20	0
	S2	16	0
August	C1	2	0
	C2	1	0
	S1	2	0
	S2	1	0

Table 5.4 Nutrient analysis for Binnimoor drain.

5.7 Discussion

The flaw in the survey method used in this investigation was that the biomass was not measured. Only the percentage coverage of each plant type could be calculated with the point-survey method that was used, although coverage, and therefore shading, will be an important competitive mechanism. The biomass of algae may be lower than the percentage coverage would suggest because algae have a lower biomass than macrophytes for a given biovolume, since there are no strengthening tissues in algae. In hindsight it would have been more reliable if a pointer could have been dropped at each 30 cm interval and a score given for each time it hit a vegetation type in that piece of the water column. In practise this would have been time-consuming and difficult to do for the entire depth of the water column, especially where growths of algae were particularly dense and/or where the water was turbid. There was no reason to suggest that the different vegetation types were unevenly spread across a transect since both the drains had flat bottoms; hence the method

used in this investigation was thought to be sufficiently accurate to determine any major algal inhibition that may have occurred in the presence of barley straw.

5.7.1 Monks Lode

In 1996 the control site had four times greater coverage of algae than macrophytes (Figure 5.3). Both the straw-treated sites had a significantly reduced algae:macrophyte ratio when compared to that of the control, although the BB site where straw had been used for two years previous to 1996 appeared to show a greater reduction in algal growth.

In 1997 the BB site was dominated by macrophytes (Figure 5.3), mainly *Elodea nuttallii* (Planchon) H. St. John and *Myriophyllum spicatum*. However, by 1997 there was no significant difference in the algae:macrophyte ratio between the AB site and the control site (Table 5.1). From Figure 5.3 the control site appears to have a higher proportion of macrophytes in 1997 than 1996 but this was not significant, which suggests there was no inhibitory effect in the AB site in 1997. The straw was accidentally taken out in 1997 so the algae were able to grow back in that year; evidently there were not enough macrophytes present from the 1996 treatment to be able to exert a controlling influence on algal growth. However, straw was only applied for one year to the AB site so presumably a three year treatment, as was applied to the BB site, could have restored macrophyte dominance.

In the BB site the lack of algae indicates that the straw was effective in suppressing algal growth, with the large percentage of macrophyte cover showing that this section appears to have been switched back to macrophyte domination. The remnants of two straw bales that were present from previous years may have been acting to suppress algal growth, but this effect was probably limited due to depletion of the straw's inhibitory potential over time (Gibson *et al.*, 1990; Ridge *et al.*, 1995). It is more likely that the macrophytes had retained dominance due to their controlling influences on algal growth (see section 5.1.1). In this case the refuge effect for phytoplankton grazers (Beklioglu & Moss, 1996; Moss *et*

al., 1998) will play little part since the bulk of algae in the drainage ditch was the filamentous *Vaucheria dichotoma*. Sequestration of nutrients by macrophytes, preventing their use by algae, was probably not significant since the nutrient levels were roughly the same in all of the sections (see Table 5.2). It is more likely that the macrophytes that were present were preventing algal growth via shading (Phillips *et al.*, 1978) and/or the release of algal growth suppressants (Scheffer *et al.*, 1993; Gross *et al.*, 1996).

5.7.2 Binnimoor drain

The results for Binnimoor drain (Figure 5.4 and Table 5.3) show that the application of barley straw had no effect at all on the ratio of algae:macrophytes compared to the controls. In 1997 the '1' sites appeared to have much more algae than the '2' sites. This is possibly due to the fact that there were large surface growths of *Enteromorpha flexuosa* in the '1' sites in 1997 which did not appear in the '2' sites, since they were prevented from flowing downstream by the culvert which separated them (see Figure 5.2). The large bloom of *E. flexuosa* did not occur in 1998, hence these growths were not recorded. Why the bloom of *E. flexuosa* did not occur in 1998 is not known, although 1998 was a cooler summer.

There could have been a number of reasons for the lack of algal inhibition in 1997. Firstly, the straw was not introduced until the end of April 1997. Since straw can take up to two months to become active (Gibson *et al.*, 1990; Ridge *et al.*, 1995), the warm weather in 1997 during this period may have produced large algal growths before the straw had become active. The growth of algae may also have been augmented by the high concentrations of nitrogen and phosphorus in Binnimoor drain compared to Monks Lode.

Secondly, dense growths of *Enteromorpha flexuosa*, which developed upstream of the treated sites, could drift downstream into the S1 site. This would produce skewed survey results and, if the amounts were large, could possibly have inactivated any inhibitors present by binding to them (Barrett, 1994). The floating mats of *E. flexuosa* did

not float past the culvert into the other sites, where no inhibition was observed, so presumably any inactivation of the inhibitors was minimal and unlikely to account for the overall lack of algal inhibition. In 1998 a floating boom was introduced upstream of the S1 site (see Figure 5.2) to prevent floating algae from drifting into the treated site.

Thirdly, the dose of barley straw may have been too low. Although the original application of 20 g m^{-3} was comparable to that shown to have had an effect at Monks Lode in 1996 (see section 5.4.1), it was much lower than the 167 g m^{-3} which had been used previously in similar sized ditches (P.R.F. Barrett, pers. comm.) or the 70 g m^{-3} used in the first two years at Monks Lode. It was for this reason that the amount of straw was increased to approximately 30 g m^{-3} in 1998.

In 1998 the ratio of algae to macrophytes was not significantly different between any of the sites. Both the '1' and '2' sites were not significantly different, possibly reflecting the fact that there were no significant surface growths of *E. flexuosa* in the '1' sites. Clearly, the introduction of more straw and the placing of a floating boom upstream of the S1 site had no effect, since no algal inhibition was observed in either of the straw treated sections.

5.7.3 Nutrients

The concentration of nitrogen and phosphorus present in the water during the winter, when no plant growth is taking place, is a good indication of the potential for algal growth (Mason, 1996). The fact that there was an algal nuisance problem at all indicated that nutrient levels in both the field sites were large enough to produce eutrophic conditions. However, nutrient analysis was carried out in 1998 to see how enriched the field sites were and to see if there were any differences between them. Data on the amounts of N and P were not available for previous years, but there was no reason to believe that nutrient levels were any different since there had been a nuisance algae problem for many years. Initially water samples were going to be taken in late January/early February so that nutrient levels during the winter could be assessed, however, this was not possible due to unforeseen

circumstances. The first samples were taken in April, hence, the nutrient levels may have been reduced due to earlier algal and macrophyte growth. $\text{NO}_3\text{-N}$ and FRP were measured, although other N and P sources such as ammonium and organic complexes were probably present.

Nutrient levels were much higher in Binnimoor drain probably because the drain is directly adjacent to arable fields and, therefore, it is likely that fertilizers were being introduced directly into the drain by accident, since farm machinery could work up to the edges of the fields. In Monks Lode 2-3 metre wide embankments on either side of the drain prevented farm machinery being used close to the drain and would have prevented direct addition of fertilizers into the drain. The embankment may also have acted as a 'buffer strip' by reducing the amount of nutrients being washed into the drain.

From Table 5.2 it can be seen that FRP levels were not particularly high in Monks Lode in April. Considering that only FRP and not total phosphorus was being measured, and the measurement was carried out after the start of the growth season, the amount of P was near the critical loading (as measured in winter) of $10 \mu\text{g P l}^{-1}$ where nuisance growths of algae can occur (Lund, 1970; Reynolds, 1984a; Harris, 1986). FRP levels in Binnimoor drain in April (Table 5.4) were well within the range where eutrophication can occur and up to five times higher than those in Monks Lode. In both sites FRP became very low or undetectable at the height of the growth season, although no data are available for Monks Lode in August. The algae were able to survive at such low FRP levels probably because they were sequestering phosphorus as soon as it entered the water column; *Vaucheria dichotoma* may also have been absorbing nutrients directly from the sediment since it forms dense tufts in close connection with the organic sediment (Cate *et al.*, 1991).

The amount of $\text{NO}_3\text{-N}$ in both sites was extremely high, especially so in Binnimoor drain, and was much higher than the critical load of $0.3 \text{ mg NO}_3\text{-N l}^{-1}$ above which algae may grow to nuisance proportions (Lund, 1970; Palmer, 1980). Although $\text{NO}_3\text{-N}$ levels were reduced at the height of the growth season they were still above the critical load,

showing that phosphorus was likely to be the limiting nutrient for algal growth, which could possibly explain the lack of phytoplankton at both sites.

The higher levels of NO₃-N could be a factor causing the lack of algal inhibition in Binnimoor drain, since they are up to four times higher than the concentrations in Monks Lode, where the straw was found to work. They are also higher than the levels found in other field studies where straw has been shown to be inhibitory to algae (Welch *et al.*, 1990; Everall & Lees, 1996). High nitrogen concentrations can retard long-term decomposition in terrestrial systems through inhibition of lignin-degrading enzymes (Berg *et al.*, 1996), which in the present situation could conceivably limit the release of algal inhibitor(s). However, NO₃-N levels in the JM1 growth medium are also extremely high at 66 mg l⁻¹ and straw has been shown to be inhibitory to a wide range of algae grown in this medium (see Chapter 3), although in the laboratory assays the barley straw was initially decomposed in aged tap water (see section 2.2) before its transfer to the JM1 medium. The barley straw may not have been inhibitory in Binnimoor drain because it was continuously in the presence of high levels of nitrogen throughout its decomposition. However, unreplicated experiments using barley straw which had been decomposed only in JM1 medium showed it still to be inhibitory to *Microcystis aeruginosa* in the laboratory (see section 4.5.1). This shows that high nitrogen levels do not retard the inhibitory action of barley straw in this case although, clearly, further experiments are required to see if the same laboratory results can be obtained for other species and whether this can be extrapolated into the field.

5.7.4 Conclusions

Why the straw did not work in Binnimoor drain is not known, although barley straw has also been found not to work in a Scottish Loch (Kelly & Smith, 1996). One of the reasons why straw does not work in certain situations is that it may not be *in situ* for long enough. Welch *et al.* (1990) reported that there was no inhibition of growth of the filamentous alga *Cladophora glomerata* during the first year of barley straw application in the highly

eutrophic Chesterfield canal. Barrett (1994) suggested that field trials should be carried out on a long-term basis, allowing at least two years after the introduction of barley straw before drawing any conclusions as to its efficacy; although straw was used in Binnimoor drain for two years but no inhibition of algal growth was observed. The only other possible reason for the lack of algal inhibition is a lack of oxidation and/or movement in the water. Binnimoor drain is smaller and much more sheltered than Monks Lode so there are fewer wind induced currents to mix the inhibitor(s). In such a situation higher doses of straw may be required to achieve any degree of inhibition. However, straw treatment of replicated experimental ponds at the Open University (OU) campus has been found not to inhibit the growth of various filamentous and planktonic algae (e.g. *Oocystis* sp., *Cladophora glomerata*, *Oedogonium* spp) (personal observation). A dose of barley straw of up to 300 g dry mass m⁻³ failed to inhibit algae in eight separate ponds at the OU over three years of application, even when bubbled vigorously with air. Barrett (1994) reported that barley straw was not inhibitory in similar experiments using polyethylene-lined tanks but that inhibitory action could be obtained in glass or fibreglass tanks (although this was found not to be the case at the OU where the ponds were made with fibreglass), which suggested that there was some form of antagonism between the polyethylene and the breakdown products released by the decomposing straw.

Clearly, any information regarding the efficacy of straw in different situations will be very important for water management and could lead to a better understanding of the algal inhibitor(s) themselves and/or their mode of action. More detailed experiments are required in the field to measure a range of physical and chemical characteristics of the water in which barley straw is applied (e.g. amounts of anions, cations, oxygen) to see under what conditions (and at what dose) straw will and will not work.

The results for Monks Lode show that barley straw can act to switch an algal dominated system to one which is dominated by macrophytes. It is questionable whether this could be classed as a switch to an alternative stable state since Blindow *et al.* (1993) suggested that studies of five years or more are required to see if the state is stable. It is

also arguable as to whether the alternative stable states, as shown by other workers (e.g. Scheffer *et al.*, 1990; Jeppesen *et al.*, 1990), are being exhibited in Monks Lode. The theory of alternative stable states in shallow waters is based on the assumption that there is a clear-water state dominated by macrophytes and a turbid-water state dominated by phytoplankton (Scheffer *et al.*, 1993). The algal growths in Monks Lode were dominated by *Vaucheria dichotoma* and very little phytoplankton growth was observed. *V. dichotoma* forms dense tufts in close connection with the organic sediment and presumably is able to absorb nutrients directly from the sediment (Cate *et al.*, 1991), although probably not as efficiently as macrophytes since it has no root system. Therefore, in both Monks Lode and Binnimoor drain, the ecology of *V. dichotoma* is somewhat analogous to macrophytes. However, the ability of *V. dichotoma* to form tightly packed mats (Dowidar & Robson, 1972; Cate *et al.*, 1991) allows it to smother macrophytes and become dominant.

From a practical point of view the return to macrophyte dominance observed in the BB site of Monks Lode will never be 'stable'. Although the algal biomass may have been reduced, the growth of macrophytes will still impede water flow (Westlake, 1975). Hence, the macrophytes will have to be removed on a regular basis, although macrophytes are easier to remove than algae (G. Cave, pers. comm.). Regular cleaning of the ditch will expose the site to recolonization by algae, so barley straw may have to be used continuously. The number of applications of straw which are needed over time, the long-term effects of straw on the ecosystem and the possibility of resistant strains of algae becoming a nuisance will all need to be investigated in further trials. However, there was no evidence of resistant strains or other nuisance species invading test sites over the short time-scale of the current surveys or in other areas where straw has been used for over ten years (P.R.F. Barrett, pers. comm.).

Observations for Monks Lode show that barley straw appears not to inhibit the growth of macrophytes, reinforcing the observations of Everall & Lees (1996), Caffrey & Monahan (1999) and those reported in Barrett (1994), who came to the same conclusion. More detailed analysis of vegetation types before and after the application of barley straw

is required to see if individual species of macrophytes are differentially affected by the inhibitory compound(s) released from decomposed plant litter.

From a practical point of view, it may be possible to use barley straw amongst a suite of techniques to help in the restoration of shallow water bodies such as lakes, and to prevent the blockage of canals and drainage channels. As well as acting to suppress algal growth leading to macrophyte regeneration, straw may also provide habitat and refugia for phytoplanktonic grazers, as has been found using bundles of alder twigs (Moss, 1987).

Chapter 6

Conclusions and suggestions for future work

The initial aims of this study (section 1.5) were divided into two main themes, laboratory assays and field studies.

(a) Laboratory studies

From carrying out assays on a wide range of algal taxa (Chapter 3), it appears that susceptibility to decomposed plant litter is not related to general taxonomic characteristics, although only a small proportion of algal taxa were assayed during the course of this work. The effects of plant litter-derived inhibitor(s) have been shown to differ widely, even amongst members of the same genus, for example, *Anabaena cylindrica* and *Oscillatoria animalis* were stimulated in the presence of litter-derived inhibitor(s) whereas *Anabaena flos-aquae* and *Oscillatoria redekei* were inhibited.

This work has shown for the first time that certain species of algae are resistant to the litter-derived inhibitor(s); in fact, most of the resistant species were actually stimulated in the presence of barley straw and oak leaf FPOM. Clearly this could have implications in the field, since it raises the possibility that the application of barley straw could actually exacerbate a nuisance algal problem. However, further work is required to see whether those algae which are found to be resistant in the laboratory are, firstly, resistant in the field and, secondly, are able to grow to nuisance proportions. Of the resistant species shown in Table 3.2, *Hydrodictyon reticulatum* is particularly interesting since it is becoming more widespread in waterbodies throughout the world (Hall & Payne, 1997; John *et al.*, 1998). The results presented in section 3.3.2 suggest that *H. reticulatum* is stimulated in the presence of barley straw, since cell expansion was greatly increased and cell division and the production of new daughter nets appeared to be unaffected. However, further investigations to determine the amount of biomass production in the presence of

litter-derived inhibitors are required to confirm the results for this potentially important alga.

Different strains of *Scenedesmus quadricauda* and *Microcystis aeruginosa* were tested but no intraspecies differences in the susceptibility to litter-derived inhibitor(s) were found. The strain of *Chlorella vulgaris* used in this investigation also appeared to require equivalent amounts of barley straw to obtain inhibition as the strain used by Gibson *et al.* (1990). The AK1 strain of *M. aeruginosa* was shown to be susceptible which, to my knowledge, has shown for the first time that a known toxin (microcystin)-producing strain of cyanobacterium is susceptible to the inhibitor(s) released from decomposed barley straw.

Ultrastructural investigations on the cyanobacterium *Anabaena flos-aquae* appear to show there is no damage to the thylakoid membranes and *Euglena gracilis* was found to be inhibited when it was grown in the dark in the presence of barley straw. Cooper *et al.* (1997) have also shown that species of *Saprolegnia*, growing heterotrophically in the dark, are inhibited in the presence of barley straw. The weight of evidence therefore suggests that the litter-derived inhibitor(s) are not acting primarily on photosynthesis but must be acting upon other physiological process(es).

When treated with litter-derived inhibitors very few species appeared to show any changes in cell or colony morphology/structure that could be observed at the level of light microscopy. Of the species which did show changes in morphology/structure these changes were found to result from the general inhibition of growth, rather than being a direct result of the litter-derived inhibitors (see Chapter 4). Electron microscopy revealed no ultrastructural differences between *Anabaena flos-aquae* and *Anabaena cylindrica*, although more detailed investigations are required to verify these findings. Cell expansion appeared to remain unaffected in most of the species tested, although cell morphology of some species was altered. Thus, the inhibitor(s) appeared to act upon the process of cell

division rather than cell expansion and there was concomitant disruption of cytoskeletal functions in some species.

(b) Field studies

Chapter five shows that barley straw can be used to control filamentous algae in the field. This supports the earlier work of Welch *et al.* (1990) with the filamentous alga *Cladophora glomerata* and the work of Barrett *et al.* (1996) and Everall & Lees (1996), who showed planktonic cyanobacterial and diatom blooms to be controlled using barley straw. The work presented in Chapter five also shows that the inhibitory action of barley straw can help to switch a system from one which is dominated by algae to one which is dominated by macrophytes. Macrophytes appear not to be affected by the algal inhibitor(s) and no resistant strains of algae were observed in the field sites over the three years of surveying, or in sites where barley straw has been used for over ten years (P.R.F. Barrett, pers. comm.).

Laboratory results appear to reflect those obtained in the field since three of the species that were shown to be susceptible to barley straw in Chapter 3 (*Asterionella formosa*, *Tabellaria flocculosa* and *Aphanizomenon flos-aquae*) were shown to be susceptible in reservoirs treated with barley straw (Barrett, *et al.*, 1996; Everall & Lees, 1996); although the dose required to achieve inhibition was much higher in the laboratory than in the field.

No algal inhibition was detected in Binnimoor drain; likewise, barley straw was found to have no inhibitory effect in a Scottish Loch (Kelly & Smith, 1996) and in replicated experimental ponds at the Open University campus (unpublished results). Clearly, any information regarding the efficacy of straw in different situations will be very important for water management and could lead to a better understanding of the inhibitory chemicals and/or their mode of action.

The use of decaying plant litter (especially barley straw) to control nuisance algae may have important implications from a water management point of view but of possibly greater significance, and largely overlooked, is the importance of these materials in natural ecosystems. Leaf litter and decaying wood are major inputs into freshwater ecosystems (Webster & Benfield, 1986) and Friberg & Winterbourne (1996) have shown that algae can be inhibited or stimulated when growing on different leaf types. Thus, natural litter inputs may influence the species composition and population growth of algal communities if conditions are favourable. Water course management in the past has largely been approached from an engineering point of view, with the aim of having clean channels and little if any riparian vegetation (Mason, 1991). It follows from this work that the presence of riparian vegetation, specifically deciduous trees, as well as providing shade to reduce excessive algal growth, may also provide plant litter inputs from which algal inhibitors may be released, provided the litter is retained under aerobic conditions in the system.

6.1 With the benefit of hindsight

With the benefit of hindsight there are a number of experiments or measurements which could have been conducted to improve certain aspects of this investigation. A number of possible improvements are listed below:

(a) The ranking of species as to their susceptibility to plant litter-derived inhibitor(s) was based upon a 50% inhibition of growth (see section 3.7.1). If growth rates had been calculated a better understanding of the growth characteristics of each species would have been gained. The algae could then have been ranked by, for example, a 50% reduction in growth rate rather than comparing 50% reductions in cell yield at the end of an assay.

The ranking could also have been improved if all the species had been assayed with decomposed oak leaf litter, thus providing more data upon which to base a ranking system. Nevertheless, the fact that all four strains of *Microcystis aeruginosa* were found to be the

most susceptible to barley straw and the fact that the strains of *Chlorella vulgaris* used here and by Gibson *et al.* (1990) were only slightly susceptible suggests that the ranking method used in this thesis was broadly correct. Overall, the results presented in this thesis do show whether a species is susceptible or resistant and therefore allow comparisons between taxa to be made.

(b) Although filamentous algae have been shown to be inhibited in the field many times, there is very little work showing inhibition of filamentous algae in laboratory assays. To my knowledge only Gibson *et al.* (1990) have performed bioassays on filamentous algae in the laboratory and they showed six different species to be inhibited by decomposed barley straw. However, the amount of straw used by Gibson *et al.* (1990) was large (10 kg dry mass m⁻³), which suggests that filamentous species are more resistant to litter-derived inhibitor(s), although filamentous species have been shown to be inhibited in the field with much less straw (e.g. Welch *et al.*, 1990; Caffrey & Monahan, 1999; the current work in chapter 5). All but one of the algal assays in this thesis (*Hydrodictyon reticulatum*) were carried out on single celled and colonial 'microscopic' species. A greater number of laboratory assays on 'macroscopic' filamentous algae would have provided more data on what are generally slower growing forms of algae. Nevertheless, the use of 'microscopic' species still allowed the first aim of the thesis to be achieved, which was to test a range of taxonomic groups.

(c) The method used to assay *Hydrodictyon reticulatum* could have been improved since the method I used did not allow comparisons of the inhibition/stimulation of biomass to be made. However, due to the growth characteristics displayed by *H. reticulatum*, whereby a large biomass can be obtained by cell expansion alone, any method to quantify biomass must take into account cell divisions as well. Attempts were made to measure the actual biomass of *H. reticulatum* by growing it in large glass tanks to allow the daughter nets to expand more fully (Pocock, 1960), however, the results were inconclusive due to growths of contaminant algae.

6.2 Suggestions for future work

The current results give few clues as to the mode of action of the inhibitor(s) and so future work concentrating on more detailed cytological investigations may provide more information as to how the inhibitor is working. The use of synchronous cultures may be a useful tool in elucidating which part of the cell cycle is being affected by the litter-derived inhibitor(s).

Although this thesis has concentrated largely on the relative sensitivity of various species of algae, more useful insights into the mode of action and/or sensitivity of species is likely to be gained by confining future investigations to specific groups of species. I would suggest that by concentrating on differences between those species which are resistant (e.g. *Anabaena cylindrica*) and those which are very susceptible (e.g. *Anabaena flos-aquae*) more useful information will be gained, especially if the species are closely related. This will prevent any ambiguity in future results, brought about by variation in the plant-litter assay, since it will dispense with the need to compare species which are similarly ranked for susceptibility. A useful addition to this may be to try and select a resistant strain of a susceptible species by growing it in increasing concentrations of decomposed plant litter; physiological comparisons can then be made between the resistant strain and the original susceptible strain.

More thorough investigations of the growth characteristics of *Scenedesmus quadricauda* are required to try and resolve the apparent paradox in its growth, whereby growth can be stimulated but the cells contain one third less chlorophyll *a* than the controls and they accumulate starch. *Euglena gracilis* also appears to be relatively less inhibited when it is grown in the light rather than in the dark (section 3.5). Investigations into (photo)heterotrophic growth may be useful for both *S. quadricauda* and *E. gracilis* to see if the ability to grow (photo)heterotrophically confers some resistance upon an alga. Investigations using abiotically liberated polyphenols from straw (Pillinger, 1993) may

prove to be useful in further investigations of (photo)heterotrophy, since this will allow axenic cultures to be maintained.

More replicated field trials would be useful in determining which algae are susceptible under 'natural' conditions and they may help to resolve why much higher masses of plant litter are required to obtain inhibition in the laboratory compared with the field. Field trials would be particularly useful for assaying the filamentous algae which do not grow well under laboratory conditions. Replicated field trials were set up during the present investigation, but barley straw was found not to be inhibitory in experimental fibreglass ponds, even when bubbled vigorously with air. Clearly, from an academic and management point of view it would be advantageous to determine under what conditions straw will and will not work. More detailed investigations of water chemistry and organic analyses, similar to those of Everall & Lees (1996 & 1997), at sites where straw is and is not working may be useful. It may be beneficial in the future to concentrate effort on finding out why the straw is not working rather than finding out how it does work.

From Table 3.2 there is a suggestion that purely planktonic species are more susceptible to litter-derived inhibitor(s) than those which are associated with surfaces. This may be an artefact of the species which were chosen for testing but it may prove useful to investigate this further to see whether the phenomenon is real.

Further evidence is required to see whether the findings presented in this thesis can be extrapolated into the field, where natural inputs of plant litter may play a part in controlling algal biomass and /or species composition in small, well aerated water bodies. Investigations surveying areas with different amounts and/or types of litter input would be useful to see if the inhibitory effects of plant litter are realized in the algal communities which exist in the field.

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Appendix 1

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Appendix 2

SI Units

Units used in text	SI units
litre	10^{-3} m^3
ml	10^{-6} m^3
psi	$6.895 \times 10^3 \text{ Pa}$
M	10^3 mol m^3
day	$8.64 \times 10^4 \text{ s}$
g	10^{-3} kg